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Effect of dietary electrolyte balance on the interplay between water fluxes and digestive functioning along the gastrointestinal tract of freshwater rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Aquaculture feed formulation is shifting from fish meal and fish oil toward other ingredients, such as plant-based ingredients, which lead to different levels and forms of minerals in diets. Dietary minerals are essential not only for growth, but also for acid-base balance and the homeostasis of fish body fluids. The gastrointestinal tract (GIT) is involved in the process of osmoregulation of salt and water during digestion, but this process can be hampered when the dietary electrolyte balance (dEB = Na - K + Cl, mEq/kg) is altered. To better understand the interaction between feeding and osmoregulation along the GIT, two diets with low (−100 mEq/kg) and high (+600 mEq/kg) dEB were designed. Freshwater rainbow trout (average initial weight, 306 g) were fed for 6 weeks. Faeces were collected during the last week for digestibility analyses. The final sampling took place over three days, with fish being sampled at 2 time points after feeding (3 and 7 h) and dissected to collect chyme from 4 GIT segments: stomach, proximal, middle and distal intestine. Chyme was analysed for dry matter, pH, osmolality, crude protein (CP) and mineral content. Yttrium oxide (Y_2O_3) was used as an inert marker to measure water fluxes, mineral fluxes, and nutrient digestibility from different gut segments. Both dEB and time after feeding altered ($p < 0.05$) chyme characteristics and water fluxes in the stomach and proximal intestine, but there was no interaction effect ($p > 0.05$). dEB also affected ($p < 0.01$) chyme pH and sodium (Na) fluxes in the stomach. Faecal digestibility of dry matter (DM) and CP was higher ($p < 0.001$) when fish were fed a high dEB diet compared to a low dEB diet. The opposite was observed for Ca availability, which was lower at the high dEB diet than at the low dEB diet ($p < 0.001$). In contrast to faecal digestibility, CP and mineral digestibility measured in the different gut segments were different predominantly in the middle and distal intestine. Our findings suggest that the GIT plays a role in regulating the alterations caused by the contrasting dEB, and that this regulation is stronger in the middle and distal intestine. Furthermore, this regulation affects nutrient and mineral digestibility in the middle and distal segments of the GIT.

1. Introduction

Due to reduced sustainability and availability of fish meal, aquaculture feed formulation is shifting toward alternative ingredients (i.e., plant-based). However, replacing fishmeal with less nutritious and digestible ingredients can changes the mineral profile of the diet and their availability. Minerals serve a variety of functions in fish, including osmoregulation and acid-base balance. When fish are fed, the acidic secretion in the stomach causes an equal amount of base secretion in the blood to maintain their body acid-base balance (Taylor et al., 2007). A change in the mineral profile of the diet can alter the dietary electrolyte balance (dEB). In animal nutrition, the dEB can be calculated in two ways, 1) considering only the monovalent ions, such as sodium, potassium, and chloride; 2) considering also the divalent ions, such as calcium, magnesium and sulfur. According to Sauvant et al. (2004), the former is better suited for monogastric animals, whereas the latter is better suited for ruminants. Therefore, in fish nutrition the dEB is often calculated as: dEB = [K + Na] - [Cl], where dEB is expressed in

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milliequivalents per kilogram (mEq/kg) of dry matter (Saravanan et al., 2013; Philip et al., 2022). A lower dEB indicates a more acidic diet, whereas a high dEB indicates a more alkaline diet (Saravanan et al., 2013). The electrolyte balance of commonly used plant-based ingredients varies widely. For example, Sauvant et al. (2004) reported a dEB for wheat gluten, soybean meal, and rapeseed meal of respectively, 708, 523, and 315 mEq/kg compared to a dEB for fishmeal of 253 mEq/kg. dEB in commercial fish feeds generally ranges between 100 and 300 mEq/kg (Tacon and De Silva, 1983; Philip et al., 2022). A low dEB may alter stomach pH and trigger compensatory mechanisms in the gastrointestinal tract (GIT). In the stomach, the compensatory mechanism consists of increased or reduced acidic secretions which might temporarily impose metabolic alkalosis or acidosis after feeding (Bucking and Wood, 2008; Lević et al., 2005; Magnoni et al., 2018). Fish use different ion transport mechanisms to correct the acid-base disturbances that occur during feeding such as branchial uptake/excretion and urinary excretion. For instance, dietary salt intake in freshwater fish may reduce branchial ions uptake or increase salt urinary excretion. Little is known about the role of the GIT in the compensation of acid-base disturbances during digestion (i.e., water fluxes, ion exchanges), since the majority of studies on osmoregulation in the GIT are done on starved fish.

However, osmoregulation can be challenged when fish are fed as water and ion secretion during digestion may disrupt fish homeostasis (Taylor and Grosell, 2006; Wood et al., 2005). In aquaculture, the diet is generally the primary source of nutrients and ions and fish have several compensatory mechanisms to prevent disturbances in ion and water balances such as water fluxes and acid-base balance. Hereby, it is still unclear how diet composition influence osmoregulation and digestive processes along the GIT and whether they interact with one another. Bucking and Wood (2006b) investigated the effect of feeding on the dynamics of water fluxes along the digestive tract of freshwater rainbow trout (*Oncorhynchus mykiss*). They observed water secretion in the stomach and proximal intestine and water absorption in the middle and distal intestine, resulting in a net loss of endogenous water. Moreover, they suggested that a net loss of endogenous water contributes to keep the internal body fluids hyperosmotic compared to the external environment. Although freshwater fish are not thought to drink because of the extremely hypotonic external environment in comparison to their internal body fluids, previous research argue that they may drink during prandial activity (Ruohonen et al., 1997; Kristiansen and Rankin, 2001). Kristiansen and Rankin (2001) showed that approximately 25–35% of the total water found in the stomach of freshwater juvenile rainbow trout came from ingesting water together with food during the consumption of a meal and approximately 34–44% came from endogenous fluid secretions.

Previous research on the effect of dEB on nutrient digestibility and chyme characteristics did not consider the effect of diet on digestion kinetics in different gut segments (Saravanan et al., 2013; Magnoni et al., 2018). Hence, there is a lack of understanding about how dEB affect the digestion kinetics along the GIT and nutrient digestibility.

Thus, the purpose of this study was to understand to what extent the GIT is involved in the overall osmoregulatory process that occurs in freshwater rainbow trout postprandially and how the dEB affects this process. In particular, we focused on the effect of contrasting dEB on chyme characteristics (dry matter, pH and osmolality) along the GIT. In addition, the relationship between chyme characteristics and nutrient digestibility as well as water and electrolyte fluxes in different segments of the GIT was studied.

2. Material and methods

This study (DEC code: 2020.W-0006.001) was performed in accordance with the Dutch law on the use of animals (Act on Animal Experiments) for scientific purposes and was approved by the Central Animal Experiments Committee (CCD) of The Netherlands. Fish were kept and handled in agreement with EU-legislation.

2.1. Experimental design

The experiment was done with mixed sex population of rainbow trout (*Oncorhynchus mykiss*) ($n = 276$) kept in freshwater. Fish were obtained from a commercial trout farm (Mohnen Aquaculture GmbH, Germany). After 2 weeks of acclimatization to recover from transportation, fish were stocked to the experimental tanks. At stocking, the fish weight was 306 ± 2.6 (mean ± SD). Fish were randomly allocated to 12 tanks with 23 fish per tank and weighted for initial weight. The experiment lasted for 6 weeks. All fish were sampled for chyme on three consecutive days (day 42–44). Fish were fed 2 experimental diets (low dEB vs high dEB) contrasting in dietary electrolyte balance (dEB). Fish performance was measured over the 42- to 44-day period. Faeces were collected the last week of the experiment to determine the apparent nutrient digestibility. Growth performance and nutrient digestibility were tested with 6 replicate tanks. During the chyme sampling days, fish final weight was measured first, and then fish were scarified either at 3 or 7 h after feeding. For parameters measured on chyme, each treatment was tested in triplicates. Final sampling is described later in more details.

2.2. Animal housing

The experiment was conducted at the aquaculture research facility (CARUS-ARF) of Wageningen University (WU). Fish were allocated to one of 12 plastic circular tanks (98 cm diameter), each with a volume of 380 l. All tanks were connected to the same recirculating water system, thus fish were kept at similar water quality conditions. The flow rate in the tanks was set to 7 l min^{-1} and the photoperiod set at 12:12 h light-dark, with daylight starting from 7:00. Water quality parameters O_2, pH, temperature, conductivity were maintained at the optimal level for rainbow trout and daily measured in the outlet water of each tank using electronic probes. The average measurements of water quality parameters during the whole trial were: O_2, 8 ± 0.7 mg/l; pH, 7 ± 0.1; temperature, 14 ± 0.3 °C and conductivity, 3 ± 0.2 mS/cm. Total ammonia nitrogen (TAN, Merck Aquamerck Colorimetric Ammonium test), nitrite (NO_2, Merck Aquamerck Colorimetric Nitrite test) and nitrate (NO_3, Merck MQuant Nitrate test strips) concentrations in the outflow were monitored three times per week and remained below 0.3 mg/l, 0.2 mg/l, and 500 mg/l, respectively.

2.3. Experimental diet

Pelleted dry feeds (floating pellets) were produced by the Research Diet Services B.V. (Wijk bij Duurstede, Netherlands) by extrusion using a win-screw extruder (Wenger, Sabetha, KS, U.S.A) with a 2 mm die size resulting in ~3 mm pellet. The experimental diets were formulated to have the same macro nutrient contents but contrasting dEB. To obtain the dietary electrolyte contrast, 1.8% of $CaCl_2$ was added to create the low dEB diet (−100 mEq/kg DM) and 2.1% of $NaCO_3$ to create the high dEB diet (+600 mEq/kg DM). Because $CaCl_2$ increases the Ca content of the low dEB diet, 1.3% of $CaCO_3$ was added to the high dEB diet to maintain equal Ca content. Diamol was added as an inert ash filler to both diets to have an equal volume of mineral mixture (Table 1). Yttrium oxide (Y_2O_3) was added as inert marker for measuring water fluxes and nutrient digestion in different gut segments. Feeds were stored at 4 °C during feeding period. Prior to feeding, feed was sieved (1.5 mm screen) to remove dust and smaller particles. A weekly sample of 100 g was collected from both diets and stored at 4 °C for analysis. Diet pH was measured under normalized conditions using a pH meter (Table 1).

2.4. Feeding

Fish were hand fed twice a day at 9:00 and 15:30 for 1 h maximum and feeding level was fixed at 1.5% of body weight/d. The amount of feed given was equal per fish and the amount of DM was equal per tank

Table 1
Ingredients and nutrient composition of the experimental diets.

	Low dEB	High dEB
Basal ingredients (%)		
Fishmeal	13	13
Wheat gluten	13	13
Soy protein concentrate	13	13
Pea protein concentrate	13	13
Wheat	27	27
Fish oil	14	14
Monocalcium phosphate	1.5	1.5
L-lysine HCL	0.3	0.3
DL-methionine	0.5	0.5
Vitamin/mineral premix[1]	1.0	1.0
Yttrium oxide	0.02	0.02
Test mineral mixture (%)		
Na_2CO_3	–	2.1
$CaCl_2.2H_2O$	1.8	–
$CaCO_3$	–	1.3
Diamol	1.9	0.3
Nutrient content (g/kg DM)		
Dry matter (g/kg)	925	955
Crude protein	444	465
Crude fat	182	174
Starch[3]	186	185
Non starch polysaccharides	87	83
Crude ash	89	89
Phosphorus	10.5	10.5
Calcium	14.8	14.7
dEB (mEq/kg DM)	−98	+600
Diet pH	5.4	7.4

dEB, dietary electrolyte balance calculated as K + Na – Cl; dEB is expressed in milliequivalents per kilogram (mEq/kg) of dry matter (Sauvant et al., 2004).

[1] Vitamin mineral premix: Vitamins (IU or mg/kg complete diet): thiamin, 10 mg; riboflavin, 10 mg; pyridoxine, 10 mg; pantothenic acid, 40 mg; niacin, 65 mg; biotin, 0.2 mg; cobalamin, 0.17 mg; folic acid, 3.3 mg; ascorbic acid, 150 mg; retinyl palmitate, 3000 IU; D-Rovimix D3–500, 2400 IU; menadione sodium bisulphite (51%), 10 mg; inositol, 400 mg; choline, 2000 mg; anti-oxidant BHT (E300–321), 100 mg; butylated hydroxytoluene, 100 mg; calcium propionate, 1000 mg. Minerals (mg/kg complete diet): iron (as $FeSO_4{\cdot}7H_2O$), 50 mg; zinc (as $ZnSO_4{\cdot}7H_2O$), 100 mg; cobalt (as $CoSO_4{\cdot}7H_2O$), 0.1 mg; copper (as $CuSO_4{\cdot}5H_2O$), 10 mg; selenium (as Na_2SeO_3), 0.2 mg; manganese (as $MnSO_4{\cdot}4H_2O$), 20 mg; magnesium (as $MgSO_4{\cdot}7H_2O$), 500 mg; chromium (as $CrCl_3{\cdot}6H_2O$), 1 mg; calcium (as $CaIO_36H_2O$), 2 mg.

[3] Starch analyses included the free sugar fraction.

for both treatments. Feeding level was calculated using fish mean initial body weight averaged over all tanks and an expected feed conversion ratio (FCR) of 0.9 to predict fish growth. Feed refusal was weighed and feed spillage was collected 15 min after feeding by settling, according to the procedure described by Amirkolaie et al. (2006) and by netting uneaten pellets out of the tank. Uneaten pellets were counted and their dry weight was estimated from the average pellet weight. Tanks were checked for mortality prior each feeding. In case of mortality, daily feeding level were adjusted based on the remained number of fish in the respective tank(s).

2.5. Sampling

Faeces were collected overnight during the last week using swirl separators connected to glass bottles. The glass bottles were submerged in ice to minimize bacterial decomposition of the faeces. Faeces were pooled per tank and stored at −20 °C for analyses to determine the digestibility. During the final sampling (day 42–44), fish were fed exactly at 3 or 7 h prior to sampling to standardize the measurement of chyme content in the GIT and to test the effect of time after feeding on chyme characteristics. The amount of feed given at the day of sampling was equal per fish basis for all treatments. Four tanks per day were sampled (2 tanks per sampling moment). All fish per tank ($n = 23$) were killed with a phenoxyethanol overdose. Chyme was collected quantitatively from four segments of the GIT: stomach, proximal, middle and distal intestine. Chyme was analysed for pH, osmolality, CP, mineral, yttrium and DM content. The collected samples were pooled per tank and stored in 250 ml plastic containers for the stomach and 150 ml plastic containers for the compartments middle, proximal and distal intestine. From these volumes a 2 ml subsample was taken for the analysis of osmolality. The total wet weight of each sample was recorded before and after subsampling, to account for this loss of chyme. Chyme pH and osmolality were measured on fresh samples using a pH-electrode SenTix SP-DIN (WTW-pH 325) and osmometer (Advanced instruments, Model 3320), respectively. Prior to analysis of mineral content, chyme samples were freeze-dried and grounded (1.2 mm coffee mill grinder). Chyme DM was calculated as the ratio between the total dry weight and the total wet weigh of the chyme (%).

2.6. Analyses and calculations

Collected faeces from week 6 were dried at 70 °C. The faeces were grounded using a mixer mill (Retsch Brinkmann; model MM2000) prior to the analysis. Collected faeces and feed were analysed for DM by drying at 103 °C for 4 h until constant weight. Ash content was determined by incineration in a muffle furnace for 4 h at 550 °C (ISO 5984, 1978). The total nitrogen content was measured using the Kjeldahl method (ISO 5983), calculating crude protein as N × 6.25. Crude fat was measured by petroleum ether extraction after acid hydrolyzes (Soxhlet method, ISO 6492) and energy by bomb calorimeter (IKA® werke, C7000; IKA analysentechnik, Weitershem, Germany). Starch including free sugar fraction in feed and faeces were determined enzymatically using amyloglucosidase without a prior ethanol extraction for removing free sugars (Goelema et al., 1998). Yttrium, P, Ca, Na and K were analysed using inductively coupled plasma mass spectrometry (ICP-MS) according to the standard method.

The apparent digestibility coefficient (ADC) of nutrients in the diets were calculated using yttrium oxide as inert marker; ADC (%) = 100 × [1 - (yttrium concentration in the feed × nutrient concentration in the faeces)/(yttrium concentration in the faeces × nutrient concentration in feed)] (Cheng and Hardy, 2003). Nutrient ADC (%) per segment were calculated as, 100 x [1 - (yttrium concentration in the feed × nutrient concentration in the chyme)/(yttrium concentration in the chyme × nutrient concentration in feed)]. Water flux (ml/g of ingested DM feed) and Na or K fluxes (mg g^{-1} of ingested DM feed) per segment were calculated as, [(relative water, Na or K content in the chyme - relative water, Na or K content in the diet)/ (relative amount of ingested feed dry matter)] according to Harter et al. (2013). The relative water (ml) or mineral (mg) content of chyme samples were calculated as, [(water or mineral content in the chyme/ marker content in the chyme)]. The relative amount of ingested feed dry matter (g DM mg^{-1} yttrium) was calculated as, [(ingested dry matter on sampling day/ yttrium content of the ingested feed)].

2.7. Performance

Growth performance was calculated as described below. The total feed intake per fish (FI, g/fish) was calculated as FI = (total DM offered feed - uneaten feed)/ (fish number). Total fish weight gain (WG, g/fish) was calculated as the difference between the average individual final (W_f) and initial (W_i) body weight per fish. The specific growth rate (SGR, %/d) was calculated as the $[\ln(W_f)-\ln(W_i))/t]*100$. The feed conversion ratio (FCR) was calculated as FI (g)/WG (g).

2.8. Statistical analyses

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). A one-way ANOVA was used to test the effect of dEB on fish growth performance, body composition, nutrient digestibility and nitrogen balance. Data of performance and digestibility were expressed as the mean per treatment of six replicates. A two-way ANOVA using a general linear model (GLM) was used for the effect of dEB and time after feeding and their interaction on chyme, water fluxes, mineral fluxes and digestion along the GIT. When interaction was found ($p < 0.05$), a Tukey HSD (honest significant difference), with multiple comparison and 95% level of significance, was used to compare treatment means. Data of chyme, water fluxes, mineral fluxes and digestion along the GIT were expressed as the mean per treatment of three replicates.

3. Results

3.1. Fish performance

Fish survival in the experiment was 98% (average over all diets) and was similar among dietary treatments ($p > 0.05$; Table 2). Fish weight gain (g/fish), SGR (%/d) and FCR were unaffected by dEB ($p > 0.05$) and mean body weight increased from 306 g to 499 g during the experiment. As intended, total feed intake per fish (g/fish) was equal between treatments ($p > 0.05$).

3.2. Chyme characteristics and water fluxes

Chyme was sampled at 2 moments postprandial. At both sampling moments, chyme was collected from 4 segments of the GIT (stomach, proximal, middle and distal intestine). All chyme characteristics (including water fluxes) were not influenced by the interaction effect between diet and sampling time ($p > 0.05$; Supplementary table S1). Therefore, we predominantly focused on the main effect of diet in this results section (Fig. 1). If significant main effects of diet and time were present on chyme parameters, this occurred only in the stomach and proximal intestine, with the latter occurring less frequently (Fig. 1, Supplementary table S1).

Averaged over diets and sampling moments, chyme DM was highest in the stomach (26.4%), whereafter it decreased and remained similar throughout the intestine, being 14.4, 15.7 and 15.5% in the proximal, mid- and distal intestine, respectively (Fig. 1). In both stomach and proximal intestine, chyme DM was lower in high dEB diet than with the low dEB ($p < 0.01$; Fig. 1A). Chyme DM in the mid- and distal intestine were similar between treatments ($p > 0.05$).

Chyme pH was the lowest in the stomach and increased as digesta passed through the intestine (Fig. 1B). Average over diets and sampling moments, chyme pH was 4.9, 7.3, 7.8, and 7.8 in stomach, proximal, middle and distal intestine, respectively. Chyme pH was only affected by dEB in the stomach ($p < 0.01$), being 4.0 in low dEB fed fish and 5.8 in high dEB fed fish.

Chyme osmolality was unaffected by dietary treatments or sampling time ($p > 0.05$; Fig. 1C) and was similar across GIT segments. Averaged over diets and time, osmolality was 392, 395, 374 and 380 mOsm kg^{-1} in stomach, proximal, middle and distal intestine, respectively.

Averaged over diets and time, the water fluxes had a positive value in the stomach and proximal intestine, indicating that water was added to the chyme in these GIT segments (Fig. 1D). Only in the stomach, water fluxes were influenced by both diet and time postprandial ($p < 0.01$), but no interaction was present (Fig. 1D, Supplementary table S1). The influx of water was higher for fish on the high dEB diet than for fish on the low dEB diet (2.6 vs 2.3 ml per g^{-1} ingested DM). In the stomach, the water influx increased between 3 h and 7 h postprandial (Supplementary table S1). Though not significant, the influx of water in the proximal intestine was numerically lower in high dEB fed fish than in low dEB fed fish (1.5 vs 1.8 ml per g^{-1} ingested DM; $p > 0.05$). Water was absorbed in the middle and distal intestine, as indicated by the negative water flux. The amount of water absorbed in these segments was similar for both dietary treatments as well as time postprandial ($p > 0.05$; Fig. 1D). The uptake of water by the intestine was largest in the middle intestine. Averaged over diets, the uptake of water was 2.5 and 0.17 ml/g ingested DM in the middle and distal intestine, respectively.

Table 2
Effect of dietary electrolyte balance (dEB) on performance of rainbow trout fed the experimental diets for 6 weeks.

	Low dEB	High dEB	SEM	*p*-value
Feed intake (g/fish)	165	161	0.53	ns
Weight gain (g/fish)	201	185	5.43	ns
SGR (%/d)	1.18	1.10	0.02	ns
FCR	0.82	0.87	0.01	ns
Survival (%)	99	97	1.11	ns

SGR, specific growth rate; FCR, feed conversion ratio on dry matter basis; SEM, standard error of mean; ns, not significant, $p > 0.05$.

3.3. Electrolyte fluxes

In all GIT segments, the fluxes of Na were unaffected by sampling moment and its interaction with diet ($p > 0.05$; Supplementary table S2). Only in the stomach, dEB influenced the Na flux ($p < 0.05$; Fig. 2A). Na was absorbed in the stomach, as indicated by the negative flux. In the stomach, fish fed the high dEB diet absorbed more Na than those fed the low dEB diet (4.5 vs −1.1 mg g^{-1} ingested feed DM; Fig. 2A). In the intestine, Na fluxes were not different between diets ($p > 0.05$). Na was secreted in the proximal intestine, whereas in the middle and distal intestine Na was absorbed again (Fig. 2A). Averaged over diets and time, the Na fluxes were 11, −9.5 and − 1.2 mg g^{-1} ingested feed DM in the proximal, middle and distal intestine, respectively.

In all GIT segments, K fluxes were unaffected by diet, sampling moment and their interaction effect ($p > 0.05$; Fig. 2B, Supplementary table S2). Potassium absorption took place in the stomach, proximal and middle intestine, while secretion of potassium occurred in the distal intestine. Averaged over diets and sampling moments, K fluxes were − 2.5, −1.4, −2.0 and 0.3 mg g^{-1} ingested feed DM in the stomach, proximal, middle and distal intestine, respectively.

3.4. Nutrient digestibility

Faecal apparent digestibility coefficients (ADC) of DM, crude protein and energy were affected by dEB ($p < 0.01$), but ADC of fat and carbohydrates were not ($p > 0.05$; Table 3). ADC of DM, crude protein and energy was higher at the high dEB diet than at the low dEB diet. In contrast, the ADC of calcium was higher in fish fed the low dEB diet compared to the high dEB diet ($p < 0.001$). The ADC of phosphorus was unaffected by dEB ($p > 0.05$).

3.5. Progression of digestion

Fig. 3 depicts the progressive ADC values until the respective GIT segment, i.e., the reported ADC for proximal intestine, is the sum of ADC in stomach and proximal intestine. Crude protein (CP) digestibility in all GIT segments were not different between 3 h and 7 h postprandial and also no interaction effect between time and diet was present ($p > 0.05$; Supplementary table S3). The ADC of CP increased from the stomach toward the distal intestine (Fig. 3A). Averaged over diets and sampling time, CP ADC was 25.3, 55.1, 86.2 and 86.6% in stomach, proximal, middle and distal intestine, respectively. In all GIT segments, ADC of CP was numerically higher for the high dEB diet, which is in line with the faecal CP digestibility. No significant differences were observed between the treatments in the different gut segments except for the middle intestine where CP digestibility increased with dEB ($p < 0.01$); being 84.5 and 87.9% for low dEB and high dEB diet, respectively.

Calcium (Ca) digestibility was affected by dEB in the stomach, middle and distal intestine and a time and a diet-time interaction effect were observed in the middle and distal intestine. Ca digestibility was

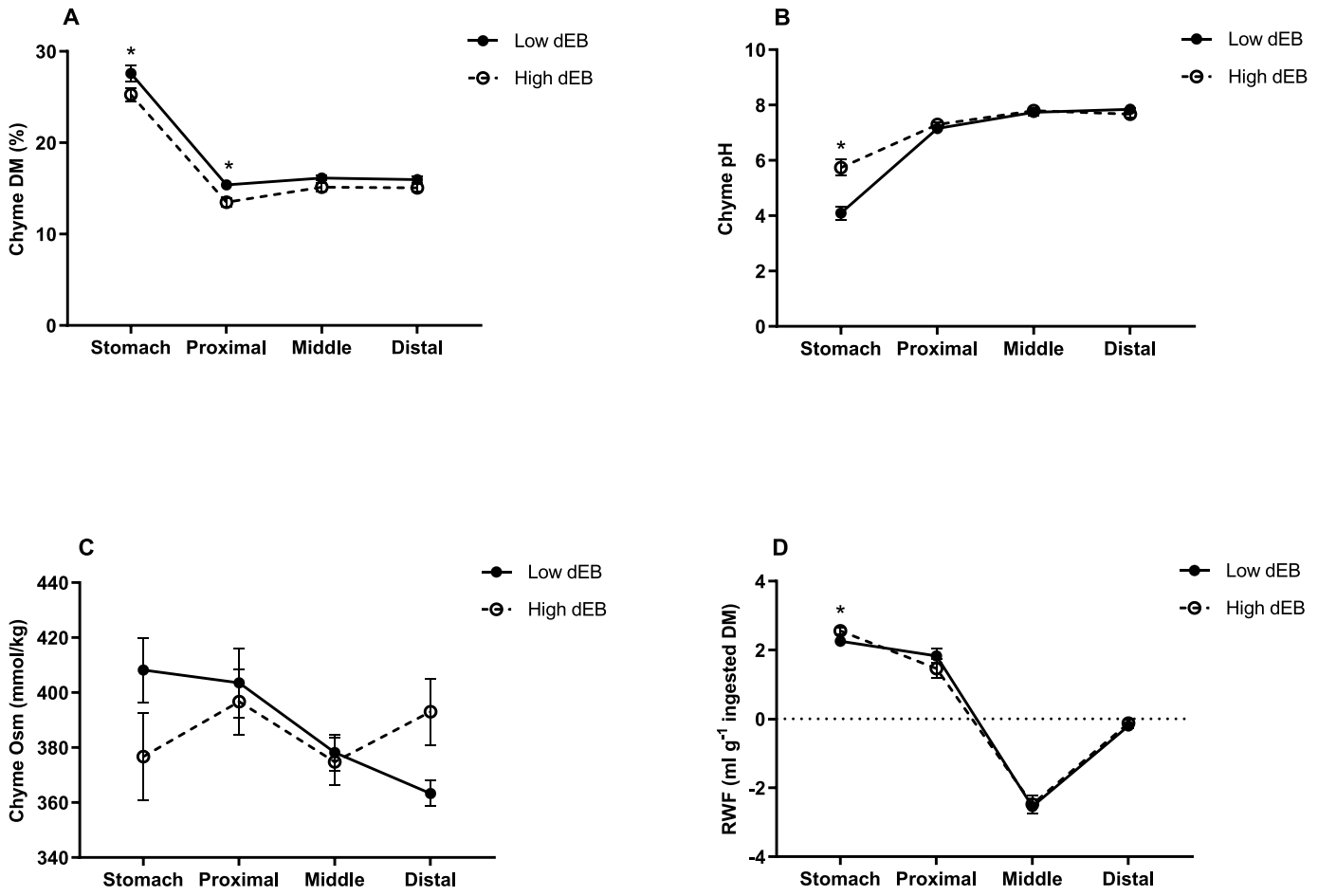

Fig. 1. Chyme parameters and relative water fluxes as affected by dietary electrolyte balance (dEB) measured in the stomach, proximal, middle and distal intestine. Values per diet are averaged over both sampling moments ($n = 6$). Figure legend: (A) chyme dry matter, DM; (B) chyme pH; (C) chyme osmolality, Osm; (D) relative water fluxes, RWF. Significant differences between diets within gastrointestinal tract segments are marked with asterisk (*) and the error bars mean the standard error mean (SEM). Mean values and level of significance are given in Supplementary table S1.

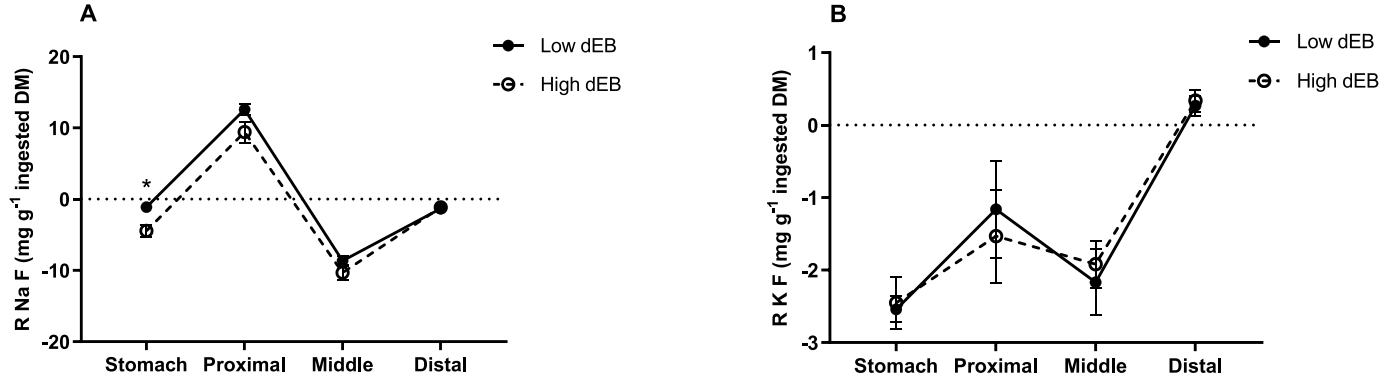

Fig. 2. Mineral fluxes as affected by dietary electrolyte balance (dEB) measured in the stomach, proximal, middle and distal intestine. Figure legend: (A) relative sodium fluxes (R Na F); (B) relative potassium fluxes (R K F); Values are means (n 6) and standard error of the mean (SEM). Significant difference between the two treatments is marked with asterisk (*).

higher in all the GIT segments in fish fed the low dEB diet compared to the high dEB diet except for the proximal intestine (Fig. 3B, Supplementary table S3). In the proximal intestine, Ca ADC were similar between diets. With progression of chyme from stomach to the proximal intestine, Ca ADC declined. Averaged over time, Ca ADC was higher ($p < 0.05$) for low dEB fed fish compared to high dEB in the stomach (22.6 vs 14.3%). In the middle and distal intestine, Ca ADC was higher ($p < 0.001$) in fish fed low dEB diet compared to the high dEB diet. In the middle intestine, the difference in Ca ADC between low and high dEB was more pronounced ($p < 0.01$) at 3 h postprandial (28.6 vs 6.8%) then at 7 h postprandial (15.2 vs 7.5%); whereas, in the distal intestine it was more pronounced at 7 h postprandial (31.5 vs 8.0%) than at 3 h postprandial (10.7 vs 9.7%).

Phosphorus (P) digestibility was unaffected by diet, time and diet-time interaction in the stomach, proximal and distal intestine (Fig. 3C, Supplementary table S3). In the middle intestine, P digestibility was

Table 3
Faecal apparent digestibility coefficients (ADC) of rainbow trout fed the experimental diets for 6 weeks.

ADC (%)	Low dEB	High dEB	SEM	p-value
DM	80.3	82.7	0.40	***
Crude protein	94.4	95.9	0.25	***
Fat	92.9	93.5	0.45	ns
Carbohydrates	67.4	66.5	0.36	ns
Energy	87.5	88.8	0.27	**
Calcium	18.8	6.9	1.84	***
Phosphorus	44	43.2	0.44	ns

dEB, dietary electrolyte balance; DM, dry matter; SEM, standard error of mean; ns, not significant, $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

enhanced ($p < 0.05$) in fish fed low dEB diet compared to high dEB diet and the effect of dEB on P ADC was more pronounced at 3 h postprandial (51.4 vs 40.9%) becoming stable at 7 h postprandial (37.0 vs 39.8%). The same trend was observed in the distal intestine but this difference was not significant, which was line with faecal P digestibility.

Sodium (Na) digestibility was affected only by diet in the intestine (proximal, middle and distal) but no diet effect was observed in the stomach (Fig. 3D, Supplementary table S3). Averaged over time, Na digestibility was negative in the proximal intestine at both diets but the magnitude was stronger ($p < 0.001$) in fish fed the low dEB diet compared to high dEB diet (−260 vs −38%). In the middle and distal intestine, the difference in Na ADC between the diets became smaller but still significant ($p < 0.001$), and the values remained negative in fish fed the low dEB diet while becoming positive in fish fed the high dEB diet (Fig. 3D).

4. Discussion

This study showed that the osmoregulatory response in the GIT may be modulated by dietary electrolyte balance. Chyme DM as well as water fluxes in different gut segments were altered by the contrasting dEB. The water influx was higher in the stomach of fish fed an alkaline diet (high dEB) compared to fish fed an acidic diet (low dEB). However, water influx was lower in the proximal intestine of high dEB fed fish. This suggests that the water flux in the proximal intestine is regulated depending on the amount of water entering the stomach. This is consistent with previous findings where water influx into the proximal intestine of African catfish decreased as a result of increased water addition to the stomach of fish fed starch diet compared to fat diet (Harter et al., 2013). In our study, water absorption in the middle and distal intestine was comparable between diets but the magnitude was higher in the middle intestine compared to the distal part which is in line with the findings of Harter et al. (2013) in African catfish. However, they reported water absorption values up to 10-fold higher in the distal intestine compared to the current study. In contrast, Bogé et al. (1988) observed water secretion in the distal intestine while studying water dynamics in the intestine of non-fed freshwater rainbow trout using an intraluminal perfusion technique. These contradictory results could be

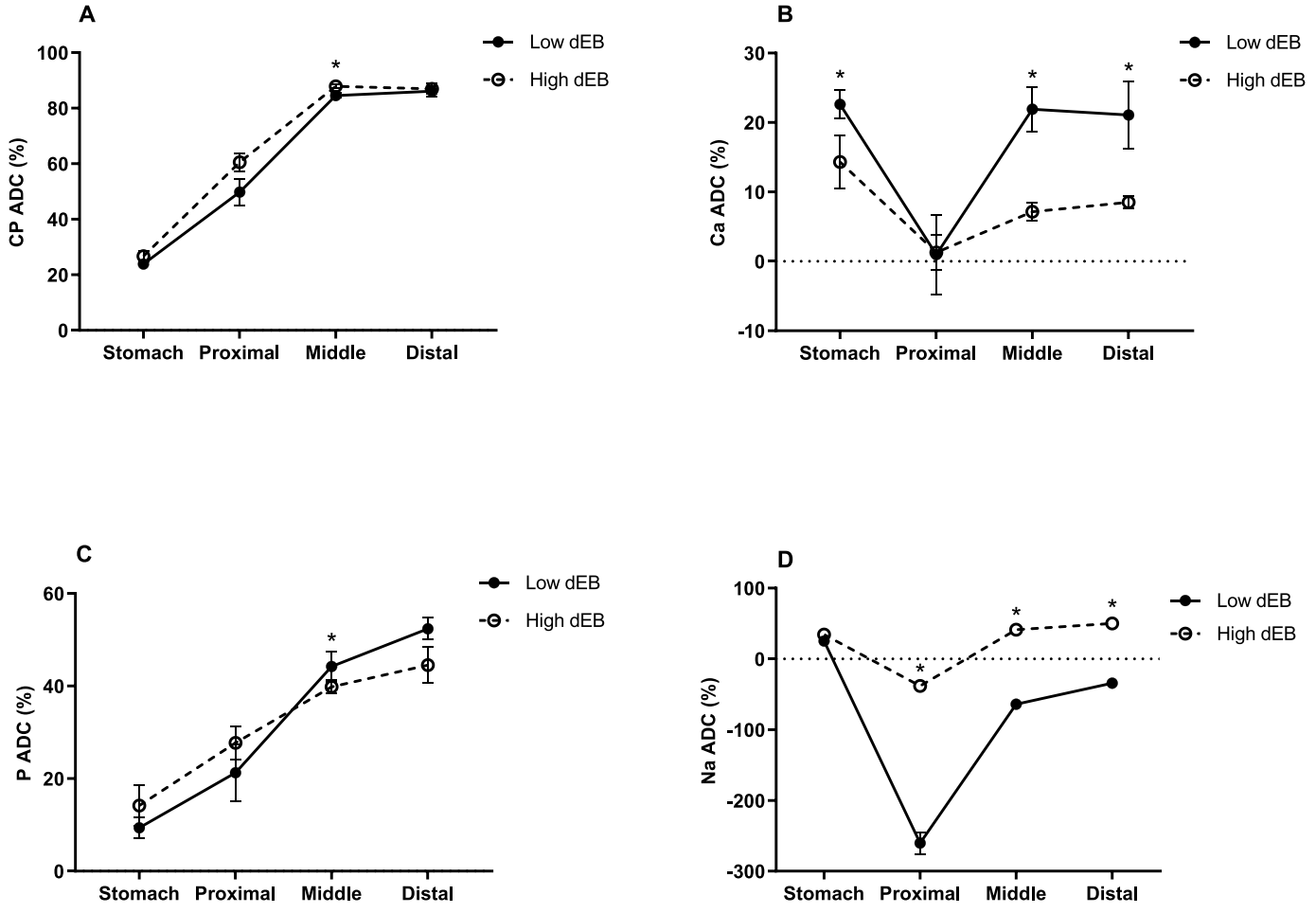

Fig. 3. Progression of digestion (ADC) as affected by dietary electrolyte balance (dEB) measured in the stomach, proximal, middle and distal intestine. Values per diet are averaged over both sampling moments ($n = 6$). Figure legend: (A) crude protein, CP; (B) calcium, Ca; (C) phosphorus, P; (D) Sodium, Na; Significant difference between the two treatment is marked with asterisk (*) and the error bars mean the standard error mean (SEM). Mean values and level of significance are given in Supplementary table S2.

attributed to the different experimental design (fed vs starved), as the presence of chyme in the lumen may alter its osmolality and water dynamics. In the current study, contrasts in dEB altered chyme characteristics and water balance predominantly in the stomach and proximal intestine, while it remained constant in the middle and distal intestine, i.e., meaning a stronger physiological regulation in the last two intestinal segments. However, when we looked at progression in digestion of CP and minerals, the effect of the diet became predominant in the latter intestinal segments. For example, an alkaline diet necessitates more acidic fluid production to decrease the pH in the stomach and enhance enzymatic digestion, resulting in increased water influx. In the current study, the increased water influx and the consequent lower chyme DM in the stomach of fish fed high dEB diet could explain the higher CP digestion observed in the middle intestine as well as in the faecal digestibility. Indeed, chyme liquefaction in the stomach promotes enzymatic hydrolysis of proteins (Saravanan et al., 2013). Previous studies likewise showed higher chyme liquefaction in the stomach along with increasing dEB resulting in higher crude protein digestibility (Saravanan et al., 2013; Magnoni et al., 2018). However, the progression of digestion of nutrients in different segments of the GIT was not measured in those studies. In contrast, Harter et al. (2015) and Elesho et al. (2022) measured the CP digestibility in different compartments of the GIT of African catfish but they did not use the dEB as a dietary factor. Both studies found that replacing dietary fat with starch resulted in decreased dry matter content, increased water influx, and increased protein disappearance in the stomach of African catfish. This is consistent with our findings, which showed that fish fed a high dEB diet had higher water influx in the stomach and increased CP digestibility along the GIT despite having a higher stomach pH. However, based on the current results, we cannot distinguish whether pH drop or water addition in the stomach is affecting protein digestibility the most. Nevertheless, based on previous research (Harter et al., 2015; Elesho et al., 2022), we propose that high dEB induced liquification enhances protein denaturation more than the low dEB induced pH drop.

Diet composition is known to influence stomach pH (Saravanan et al., 2013; Magnoni et al., 2018). In the current study, stomach pH was found to be the lowest in fish fed a low dEB diet. The current findings can be related to the electrolyte fluxes: when fish were fed a more alkaline diet, a greater amount of sodium was taken up from the stomach as a result of pH regulation. In fact, sodium monovalent cations contribute to the formation of a basic environment in the stomach lumen, so Na^+ absorption may lower the pH in the stomach. Furthermore, when Na^+ is absorbed, the osmotic pressure in the gut lumen rises, stimulating water absorption (Bogé et al., 1988; Bucking and Wood, 2006a; Skadhauge, 1974). Despite increased Na^+ absorption, water secretion was higher in the stomach of fish fed the alkaline diet which required more acidic secretion to lower the pH. However, stomach pH of high dEB fed fish was higher compared to the low dEB fed fish. This suggests that fish do not completely compensate for the increasing effect of dEB on stomach pH. The effect of dEB on chyme pH appears to be consistent with findings in Nile tilapia (Saravanan et al., 2013) and rainbow trout (Magnoni et al., 2018), where stomach pH increased with dEB. Moreover, low dietary dEB can lower the pH in the stomach enhancing the activity of proteolytic enzymes and protein denaturation (Bakke et al., 2010). In this experiment, however, protein digestibility did not improve with a low dEB diet. In contrast, crude protein digestibility increased with high dEB as well as water influx in the stomach. As mentioned above, an alkaline diet requires more HCl secretion to lower the pH of the stomach. The release of HCl in the stomach stimulates protein denaturation by the proteolytic enzyme pepsin, converting stomach content to chyme, which moves into the intestine for further digestion and absorption (Bakke et al., 2010). In the current study, the positive CP digestibility in the stomach is possibly an artefact of solubilized proteins (peptides/amino acids) transiting to the intestine earlier than yttrium due to nutrient sensing driven peristalsis. In contrast, the digestibility of minerals occurs also in the stomach and can be enhanced by a lower pH (Sugiura et al., 2006). In the current study, the acidic diet had a beneficial effect on calcium digestibility in all gastrointestinal segments. However, calcium digestibility decreased in the proximal intestine for both dietary treatments. This value can be explained by the presence of a calcium source coming from gall bladder bile acid secretions (Grosell et al., 2000). Bile acid secretions can likewise explain the positive sodium flux as well as the negative sodium digestibility observed for both dietary treatments in the proximal intestine. The postprandial sodium secretion in the proximal intestine accounts for about 155 mol/ml (Dabrowski et al., 1986; Grosell et al., 2000). Bucking and Wood (2006a) made the same observation and proposed that gall bladder bile acid secretions could explain, at least in part, the simultaneous significant secretions of Na in the proximal intestine of rainbow trout during digestion. In accordance with the present results, they also observed Na absorption in the middle and distal intestine. In contrast, when measuring Na digestibility in the middle and distal intestine, our results contradict what we observed in the fluxes. Indeed, Na digestibility was negatively affected by the low dEB diet in the middle and distal intestine. This observation demonstrate that the dEB affects the digestive physiology of fish intestine even though this is not shown when looking at the chyme characteristics in the final parts of the gut (middle and distal intestine).

The Na flux is linked to the K flux by the Na/K/Cl-co-transporter (NKCC2) and it is carried out by the Na/K-ATPase (NKA) (Grosell, 2010). Despite the presence of a small amount of K in bile acid secretions (8 ml/l) (Bucking and Wood, 2006a), dietary K was almost completely absorbed from the stomach to the intestine, with the exception of a very small concentration of K secretion in the distal part, which could be endogenous or exogenous. Likewise, Bucking and Wood (2006a) found that when chyme moved from the stomach to the proximal intestine, where bile is secreted, the relative K concentration did not alter significantly. However, they reported a net K absorption in all GIT segments which is in contrast with what was found in the current study where K secretion occurred in the distal intestine. K influx requires the presence of luminal Na, and its secretion in the distal intestine might be a consequence of the lower Na absorption.

In conclusion, our findings show that contrasting dEB alters chyme characteristics and water dynamics primarily in the stomach and proximal intestine, but they stabilize in the middle and distal intestine, implying that osmoregulation is stronger in the latter part of the GIT. When studying the progression of digestion (CP, minerals), differences due to contrasting dEB reappear in the final intestinal segments, indicating the presence of a physiological response of the fish to the homeostatic disturbance caused by the diet. Because we only measured two contrasting levels of dietary electrolyte balance, it is difficult to recommend an optimal dEB level for trout based on our findings. To discuss an optimal dEB level for trout, a dose response study is required.

CRediT authorship contribution statement

Elisa Ciavoni: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Roel M. Maas:** Methodology, Validation, Formal analysis, Writing – review & editing, Supervision. **Marc Koppelaars:** Methodology, Formal analysis, Investigation. **Øystein Sæle:** Conceptualization, Methodology, Writing – review & editing. **Johan W. Schrama:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition. **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.

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Appendix A. Supplementary data

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