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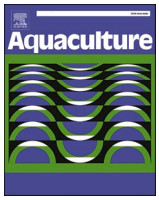

Effect of dietary macronutrient composition and buffering capacity on chyme characteristics and digestion kinetics in the gastrointestinal tract of freshwater rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

The aim of this study was to investigate the impact of dietary macronutrient composition and buffering capacity (BC) on chyme characteristics and digestion kinetics in freshwater rainbow trout (*Oncorhynchus mykiss*). Dietary macronutrient composition was altered by changing the protein-to-energy ratio (P:E) while keeping the fat-to-starch ratio constant. Dietary BC was increased by supplementation of $CaCO_3$. The experiment lasted for 6 weeks. Fish were fed four diets having high and low P:E ratio and high and low $CaCO_3$ level. This experiment was planned according to a 2x2x2 factorial design. The three factors were dietary P:E ratio, BC and time sampling after feeding (3 and 7 h). Chyme was collected from four gastrointestinal tract (GIT) segments (stomach, proximal, middle and distal intestine) and analysed for dry matter (DM), pH, osmolality, crude protein (CP) and mineral content. Relative water fluxes (RWF), electrolyte fluxes, kinetic of digestion and faecal digestibility (ADCs) were measured using yttrium oxide (Y_2O_3) as an inert marker. All chyme characteristics (including water fluxes) were not influenced by the interaction effect between dietary factors and sampling time ($p > 0.05$). Both dietary treatments did not affect chyme DM in the stomach. Low P:E diet increased ($p < 0.001$) chyme DM in all the intestinal segments. Dietary $CaCO_3$ only affected ($p < 0.05$) chyme DM in the distal intestine. Low P:E diet decreased ($p < 0.001$) chyme pH in all GIT segments compared to the high P:E diet. Low $CaCO_3$ diet decreased chyme pH in the proximal and middle intestine ($p < 0.05$) compared to the high $CaCO_3$ diet. RWF were affected only by the dietary P:E ratio in the stomach and in the proximal intestine. Fish fed the high P:E diet had a lower water influx in the stomach and a higher water influx in the proximal intestine than fish fed the low P:E diet. Dietary P:E ratio affected electrolyte fluxes in the GIT, while no effect of $CaCO_3$ was detected. Both dietary factors had a minimal or no effect on the kinetic of digestion in the different GIT segments, while a significant effect was present in all ADCs. Our findings suggest that dietary macronutrient composition, rather than buffering capacity, is the primary factor responsible for changes in chyme characteristics, water and ion fluxes in the GIT of freshwater rainbow trout. Furthermore, changes in dietary macronutrient composition and buffering capacity significantly affect faecal digestibility but are not reflected in digestion kinetics.

1. Introduction

The ingredient/nutrient composition of the feed determines its buffering capacity (BC). BC is defined as the ability of the feed to withstand a pH change after adding an acidic or basic solution (Giger-Reverdin et al., 2002). Feed BC is affected by its composition (protein, fat, minerals, and organic acids) as well as properties (i.e., physical state and particle size). Protein-rich ingredients like soybean meal, fishmeal, or milk powder have a higher BC than cereals like corn, wheat, or barley (Levic et al., 2005). Feeds with a high mineral concentration have a higher BC. Generally, plant ingredients have a lower BC than fish meal (Giger-Reverdin et al., 2002; Parma et al., 2019). Thus, the BC of a feed is determined by the combination and inclusion levels of the ingredients in the formula. Changes in dietary BC may alter the physiological conditions in the gastrointestinal tract (GIT) and thereby affect the chyme characteristics (i.e., chyme dry matter, pH, osmolality and water fluxes)

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during digestion. As a result, the physicochemical breakdown of pellets and nutrient digestibility may be altered. The optimal acidity for pepsin activity in the stomach of most cultured fish species is between pH 1.5 and 3.4 (Krogdahl et al., 2015; Sugiura et al., 2006). Feed components with higher buffering capacity may negatively affect enzymatic digestion by increasing stomach chyme pH. Fish feeds can be formulated to reduce chyme pH in the stomach and promote nutrient digestion. Therefore, understanding the relation between dietary BC and digestive characteristics (chyme properties, water/nutrient fluxes) along the GIT of fish is crucial. Dietary BC has been mainly studied in ruminants, swine and humans for in vitro and in vivo studies on nutrient digestibility and growth performance (Mennah-Govela et al., 2019; Lückstädt et al., 2004; Mroz et al., 2000). Mroz et al. (2000) used organic acid to lower the dietary BC, which reduced chyme pH in the stomach of swine and promoted nutrient digestibility. A recent study of Goodrich et al. (2022b) showed that juvenile barramundi (*Lates carcarifer*) fed acidified diets by adding HCl reduced endogenous acid secretion in the stomach. However, information on the effect of dietary BC on nutrient digestibility and chyme characteristics in fish is limited. Parma et al. (2019) observed that adding calcium carbonate ($CaCO_3$) to the diet increased dietary BC, but did not affect chyme pH and enzymatic activity in the stomach of European sea bass (*Dicentrarchus labrax*). However, further effect of dietary BC on water fluxes and nutrient digestibility has not been studied.

Changing macronutrient composition in the diet can affect chyme characteristics and digestion kinetics along the GIT. Elesho et al. (2022) and Harter et al. (2015) observed that chyme dry mater (DM), water fluxes and digestion kinetics were affected by the type of non-protein energy source (NPE) present in the diet of African catfish (*Clarias gariepinus*). When keeping the protein-to-energy ratio (P:E) constant, they observed that replacing dietary fat by starch resulted in higher stomach chyme DM and lower water influx in the stomach. Moreover, the replacement of dietary fat by starch as a source of NPE lowered the faecal crude protein digestibility (CP ADC) in African catfish (Harter et al., 2015). Because starch is less expensive than fat, it is increasingly being used as an energy substitute in aquaculture feed formulation (Harter et al., 2015), but it can be a limiting factor for nutrient digestibility when the inclusion level increases in the diet of carnivorous fish species as Atlantic salmon (*Salmo salar*) (Arnesen et al., 1995; Grisdale-Helland and Helland, 1997; Hillestad et al., 2001; Krogdahl et al., 1999), rainbow trout (*Oncorhynchus mykiss*) (Bergot and Breque, 1983) and cod (*Gadus morhua*) (Hemre and Lambertsen, 1989). However, the effect of starch on nutrient digestibility differs depending on the source of starch, fish species and starch processing method (i.e. native versus gelatinized). In a comparative study between rainbow trout and Atlantic salmon, Krogdahl et al. (2004) observed that high dietary inclusion levels (23%) of pre-cooked maize starch reduced nutrient digestibility in both fish species compared to low dietary starch inclusion (7%). Extensive research on nutrient digestibility has primarily focused on replacing fat with starch (mostly gelatinized), but the impact of protein level versus non-protein energy source has not been investigated. Furthermore, as more information about fish nutritional requirements and optimal amino acid profile became available, the protein content of diets has been reduced over time (National Research Council, 2011). However, information on the effect of P:E ratio on chyme characteristics and digestion kinetics along the GIT is limited in fish.

The aim of the current study was to investigate the impact of dietary macronutrient composition and buffering capacity on chyme characteristics and kinetics of digestion along the gastrointestinal tract of freshwater rainbow trout (*Oncorhynchus mykiss*). In this study, we focused on changing the dietary macronutrient composition by altering the protein-to-energy ratio and keeping the fat-to-starch ratio constant. Dietary buffering capacity was altered by supplementation of $CaCO_3$.

2. Material and methods

This study (DEC code: 2020.W-0006.002) 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 and diets

This experiment was planned according to a 2x2x2 factorial design. The first factor was dietary protein-to-energy ratio (P:E). The P:E contrast was created by exchanging a protein mixture consisting mostly of wheat gluten, soya protein and pea protein concentrate, with an energy mixture consisting of gelatinized maize starch, wheat and fish oil (Low P:E versus High P:E). The second factor was dietary buffering capacity (BC). Dietary BC was studied at two levels by adding 0 versus 3% $CaCO_3$ to the low or high P:E basal mix (Table 1). The third factor was chyme sampling moment at the end of the experiment. Chyme was sampled at 3 and 7 h postprandial from the gastrointestinal tract (GIT) segments (stomach, proximal, middle and distal intestine). These sampling moments were used to standardize the measurement of chyme content in the GIT and to determine whether the effect of diet on chyme characteristics changes over time. Yttrium oxide (Y_2O_3) was added to all the diets as inert marker to measure nutrient digestibility and water fluxes in the GIT segments. Pelleted dry feeds (sinking pellets) were produced by the Research Diet Services B.V. (Wijk bij Duurstede, Netherlands) by extrusion using a twin-screw extruder (Wenger, Sabetha, KS, U.S.A) with a 2 mm die size resulting in ~3 mm pellet. Feeds were stored at 4 °C prior to feeding. 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.

2.2. Experimental animals and housing

The experiment was done with a mixed sex population of rainbow trout (*Oncorhynchus mykiss*) (n = 552) kept in freshwater. Fish were obtained from a commercial trout farm (Mohnen Aquaculture GmbH, Germany). The experiment was conducted at the aquaculture research facility (CARUS-ARF) of Wageningen University (WU), The Netherlands. After 2 weeks of acclimatization to recover from transportation, fish were stocked into the experimental tanks (98 cm diameter), each with a volume of 380 L. At stocking, the mean fish weight was 284 ± 2.5 g (mean ± SD) and were randomly distributed to the tanks at 23 fish per tank. All tanks were connected to the same recirculating water system. Thus, the fish were kept at similar water quality conditions. The flow rate into 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 using electronic probes. The average measurements of water quality parameters during the whole trial were: O_2, 7.5 ± 0.7 mg/L; pH, 8 ± 0.2; temperature, 14 ± 0.2 °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 feeding

The 24 tanks were randomly allocated to one of the four experimental diets (6 replicates per treatment). Fish were hand fed for 6 weeks. Feeding was done 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 tank based on the number of fish (on DM basis).

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

	Low P:E Basal mix		High P:E Basal mix	
Protein mixture (%)				
Wheat gluten	12		21	
Soya protein concentrate	12		21	
Pea protein concentrate	12		21	
L-Lysine HCl	0.4		0.6	
DL-Methionine	0.5		0.7	
L-Threonine	0.3		0.4	
Energy mixture (%)				
Gelatinized maize starch	20		9	
Wheat	20		9	
Fish oil	13		6	
Other ingredients (%)				
Fish hydrolysate	2		2	
Vitamin mineral premix[1]	1		1	
Monocalciumphospate	3		3	
$CaCl_2{\cdot}2H_2O$	0.5		0.5	
Yttrium oxide	0.02		0.02	
	Low P:E Low $CaCO_3$	Low P:E High $CaCO_3$	High P:E Low $CaCO_3$	High P:E High $CaCO_3$
Basal mix	100	97	100	97
$CaCO_3$	0	3	0	3
Nutrient content (g/kg DM)				
Dry matter	943	931	928	939
Crude protein	357	346	575	556
Crude fat	173	167	114	114
Carbohydrates	416	410	246	243
Starch[3]	366	363	205	196
Crude ash	53.5	76.6	64.9	86.7
P	10.7	10.4	11.6	11.6
Ca	7.8	19.4	7.7	19.5
Na	2.9	2.7	4.6	4.2
K	4.8	4.5	6.5	6.4
GE (mg/kg DM)	22.2	21.4	21.8	21.1
CP:GE (mg/kJ)	16.1	16.2	26.4	26.4
DP:DE (mg/kJ)	17.9	18.0	28.3	28.2
Dietary starting pH and buffering capacity				
pH	5.96	6.36	5.75	6.43
Acidic BC (mmol HCl/g feed)[4]	0.212	0.328	0.284	0.406
Alkaline BC (mmol NaOH/g feed)[5]	0.174	0.218	0.230	0.260

P:E, protein to energy ratio; Ca, calcium level; CP:GE, crude protein to gross energy ratio; DP:DE, digestible protein to digestible energy ratio; Acidic BC, acidic buffering capacity; Alkaline BC, alkaline buffering capacity.

[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; d-alpha-tocopherol, 200 IU; 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; calcium propionate, 1000 mg.

Minerals (mg/kg complete diet): iron (as $FeSO_4{\cdot}7H_2O$), 50 mg; zinc (as $ZnSO_4{\cdot}H_2O$), 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.

[4] Amount of HCl needed to lower the pH from the dietary start pH to pH = 3.

[5] Amount of NaOH needed to increase the pH from pH 3 to pH = 8.

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 spillage was collected 15 min after feeding by settling according to the procedure described by Amirkolaie et al. (2006) and by netting uneaten floating pellets out of the tank to calculate feed intake. Uneaten pellets were counted and their dry weight was estimated from the average pellet weight. Tanks were checked for mortality prior to each feeding. In case of mortality, the feeding level were adjusted based on the remained number of fish in the respective tank(s).

2.4. Sampling

Faeces were collected overnight for 5 days during the last week of the experiment (week 6). This was done by using swirl separators to which glass bottles were connected. To prevent bacterial decomposition of the faeces, the bottles were submerged in ice water. Faeces were pooled per tank and stored at −20 °C for digestibility analysis.

At the end of week 6, the final sampling was spread over three days to collect chyme (days 42–44), because of being labor intensive. Eight tanks were sampled per day (4 tanks per sampling moment). An overdose of phenoxyethanol (1 mL/L) was used to kill the fish. Chyme was collected quantitatively from four segments of the GIT: stomach, proximal, middle and distal intestine. Before collecting the chyme, clippers were placed in the junctions of the different segments to ensure that the contents were not mixing. The method was adapted from Bucking and Wood (2009). The collected samples were pooled by tank and stored in plastic containers. A subsample was collected from these volumes and placed in a 2 mL Eppendorf tube for osmolality analysis. To account for chyme used for osmolality measurements, the total wet weight of each sample was recorded before and after subsampling.

2.5. Analytical methods

Dietary acidic and alkaline BC were determined using a pH-stat method modified from Prohászka and Baron (1980). For both measurements, 100 mg of feed sample were dissolved in 10 mL of water and kept at 37 °C for 1 h. To determine the acidic buffering capacity, 1 M of HCl was added to the sample to adjust the pH from the initially measured pH to pH 3. To determine the alkaline buffering capacity, 1 M of NaOH was added to the sample to raise the pH from 3 to pH 8 (Table 1).

Collected faeces during week 6 were oven dried at 70 °C. The faeces were grinded 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 24 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), and crude protein calculated as N × 6.25. Crude fat was measured by petroleum ether extraction after acid hydrolyzes (Soxhlet method, ISO 6492) and gross energy by bomb calorimeter (IKA® werke, C7000; IKA analysentechnik, Weitershem, Germany). Total starch was analysed enzymatically using amyloglucosidase after washing with 40% ethanol (Zhu et al., 2016). Yttrium, P, Ca, Na and K were analysed using inductively coupled plasma mass spectrometry according to the standard NEN-EN-ISO 11885:1998.

Chyme was analysed for pH, osmolality, crude protein, mineral, yttrium and dry matter content. Chyme pH was determined on fresh sample using a pH-electrode SenTix SP-DIN (WTW-pH 325). After collection, a subsample of chyme was centrifuged (3500 rpm for 5 min at 4 °C) to sample the liquid phase and measure chyme osmolality using an osmometer (Advanced Instruments, Model 3320). The chyme samples were freeze-dried to obtain dry matter and ground (1.2 mm coffee mill grinder) before being analysed for mineral and protein content. Yttrium, P, Ca, Na and K were analysed using inductively coupled plasma mass spectrometry according to the standard NEN-EN-ISO 11885:1998. Protein was analysed using the Dumas method (Nielsen, 2017).

2.6. Calculations

Fish performance was measured over the 42- to 44-day period. Growth performance was calculated as described in Saravanan et al. (2013). The feed intake per fish (FI, g/fish) was calculated as FI = (total offered feed - uneaten feed)/ (number of fish) (on DM basis). The weight gain (Wg, g/fish) was calculated as the difference between the average individual final (Wf) and initial (Wi) body weight per fish. The specific growth rate (SGR, %/d) was calculated as the (ln(Wf) - ln(Wi))/t)*100.

The feed conversion ratio (FCR, on DM basis) was calculated as FI/Wg.

Nutrient apparent digestibility coefficients (ADCs) in the faeces were calculated using yttrium oxide (Y_2O_3) 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 ADCs (%) per segment were calculated as, 100 x [1 - (yttrium concentration in the feed × yttrium concentration in the chyme)/ (nutrient concentration in the chyme × nutrient concentration in feed)].

The water and mineral fluxes in the chyme were calculated relative to the marker content (yttrium). According to Harter et al. (2013), relative water and ion fluxes (Ca, Na and K) were calculated per segment of the GIT: stomach, proximal, middle and distal intestine. In the stomach, relative water flux (mL g^{-1} of ingested DM feed) and ion fluxes (mg g^{-1} of ingested DM feed) were calculated as, [(relative water or ion content in the stomach chyme - relative water or ion content in the diet)/ (relative amount of ingested feed dry matter)]. In the proximal, middle and distal intestine, water and ion fluxes were calculated as, [(relative water or ion content in the chyme of the intestinal segment - relative water or ion content in the chyme of the previous segment)/ (relative amount of ingested feed dry matter)].

2.7. 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 two-way ANOVA was used to test the effect of dietary P:E ratio, dietary $CaCO_3$ level and their interaction on fish growth performance and nutrient ADCs. A three-way ANOVA by means of a general linear model (GLM) was used to test the effect of dietary P:E ratio, dietary $CaCO_3$ level, time after feeding (3 and 7 h) and their interactions on chyme DM, pH, relative water fluxes, electrolyte fluxes and kinetic of digestion in the GIT of rainbow trout. When an interaction effect was significant ($p < 0.05$), a Tukey HSD (honest significant difference), with multiple comparison and 95% level of significance was used to compare treatment means. However, the effect of time and the interaction effect of time with the dietary factors was almost absent on all analysed parameters. Therefore, in all figures and tables presented in the result section, the values were presented as the main effect of dietary P:E ratio or $CaCO_3$ level. The individual means for time point and their interaction effects can be found in the Supplementary tables S1, S2 and S3. Figures were made using GraphPad Prism version 8.

3. Results

3.1. Fish performance

Fish performance is depicted in Table 2. All diets were readily consumed and feed intake (g DM/fish) was similar between treatments ($p > 0.05$). Fish mean body weight increased from 284 g to 564 g during the experiment. Weight gain (g/fish) and SGR (%/d) were higher in fish fed the high P:E diet ($p < 0.001$), while FCR was lower compared to the low P:E diet ($p < 0.001$). High $CaCO_3$ diet fed fish had lower growth and higher FCR ($p < 0.01$) than low $CaCO_3$ diet fed fish. Averaged over all diets, fish survival was 99%.

3.2. Chyme characteristics and relative water fluxes

Chyme was sampled at two moments postprandial and collected from four segments of the GIT (stomach, proximal, middle and distal intestine). The effect of time and the interaction effect of time with the dietary factors was almost absent on chyme characteristics (dry matter and pH) and relative water fluxes (RWF) in all GIT segments. Therefore, only the dietary effects are depicted in Fig. 1. The full 3-way ANOVA results with all treatments means including sampling time is reported in the Supplementary table S1.

DM contents of all diets were similar, ranging between 92 and 94% (Table 1).

Chyme DM (Fig. 1A, D) was not affected by dietary treatments in the stomach and, averaged over diets and sampling moments, chyme DM was 32.5% (Supplementary table S1). In contrast to the stomach, P:E ratio altered ($p < 0.001$) chyme DM in all the intestinal segments (Fig. 1A). Averaged over the three intestinal segments, chyme DM was 18.7% and 15.8% in fish fed the high and low P:E diets, respectively. The differences in chyme DM between contrasting $CaCO_3$ diets were much smaller compared to the main effect of P:E ratio (Fig. 1D). Dietary $CaCO_3$ only affected ($p < 0.05$) chyme DM in the distal intestine; being 16.0% and 17.1% in fish fed the low and the high $CaCO_3$ diets, respectively.

Averaged over both $CaCO_3$ diets, the pH of the low and high P:E diets was 6.2 and 6.1, respectively and acidic dietary BC was 0.270 and 0.345 mmol HCl/g sample, respectively (Table 1). Averaged over both P:E ratio diets, the pH of the low and high $CaCO_3$ diets was 5.9 and 6.4, respectively and acidic dietary BC was 0.248 and 0.367 mmol HCl/g sample, respectively. The alkaline dietary BC was similar between all treatments (Table 1). In the stomach, chyme pH was not affected ($p > 0.05$) by dietary $CaCO_3$ supplementation (Fig. 1E). In contrast, dietary P:E ratio had an effect ($p < 0.001$) on chyme pH in the stomach, which was lower in fish fed the low P:E diet compared to the high P:E diet (4.2 versus 4.7) (Fig. 1B). Both dietary factors affected chyme pH in the proximal and middle intestine, but no effect was detected in the distal intestine. Chyme pH increased ($p < 0.001$) between the low and the high P:E diet in the proximal (7.0 versus 7.2) and middle intestine (7.7 versus 7.9) (Fig. 1B). Chyme pH increased ($p < 0.05$) between the low and the high $CaCO_3$ diet in the proximal (7.0 versus 7.2) and middle intestine (7.7 versus 7.9) (Fig. 1E).

Due to the relatively high DM content in the stomach, we were unable to measure chyme osmolality in the liquid phase. Therefore, chyme osmolality was only measured in the intestinal segments and it was unaffected by both dietary treatments (Supplementary table S1).

Overall, relative water fluxes (RWF) had a positive value in the stomach and proximal intestine, and a negative value in the middle and distal intestine, indicating water influx to the former segments and water absorption from the latter (Supplementary table S1). Dietary $CaCO_3$ had no effect on RWF in all GIT segments (Fig. 1F). In contrast,

Table 2
Effect of contrasting levels of protein to energy ratio (P:E) and calcium carbonate ($CaCO_3$) on performance of rainbow trout fed the experimental diets for 6 weeks.

	Low P:E		High P:E			*p-values*		
	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	pSEM	P:E	$CaCO_3$	P:E* $CaCO_3$
Feed intake (g/fish)	244	246	248	244	0.03	ns	ns	ns
Weight gain (g/fish)	271	259	302	284	4.02	***	**	ns
SGR (%/d)	1.58	1.53	1.68	1.60	0.02	***	**	ns
FCR	0.83	0.90	0.77	0.82	0.02	***	**	ns
Survival (%)	100	98	99	100	0.62	–	–	–

SGR, specific growth rate; FCR, feed conversion ratio on dry matter basis; pSEM, pooled standard error of mean; ns, not significant, $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The data were analysed by a 2-way ANOVA using dietary P:E ratio and $CaCO_3$ level as independent factors. Values are expressed as the mean per treatment ($n = 6$).

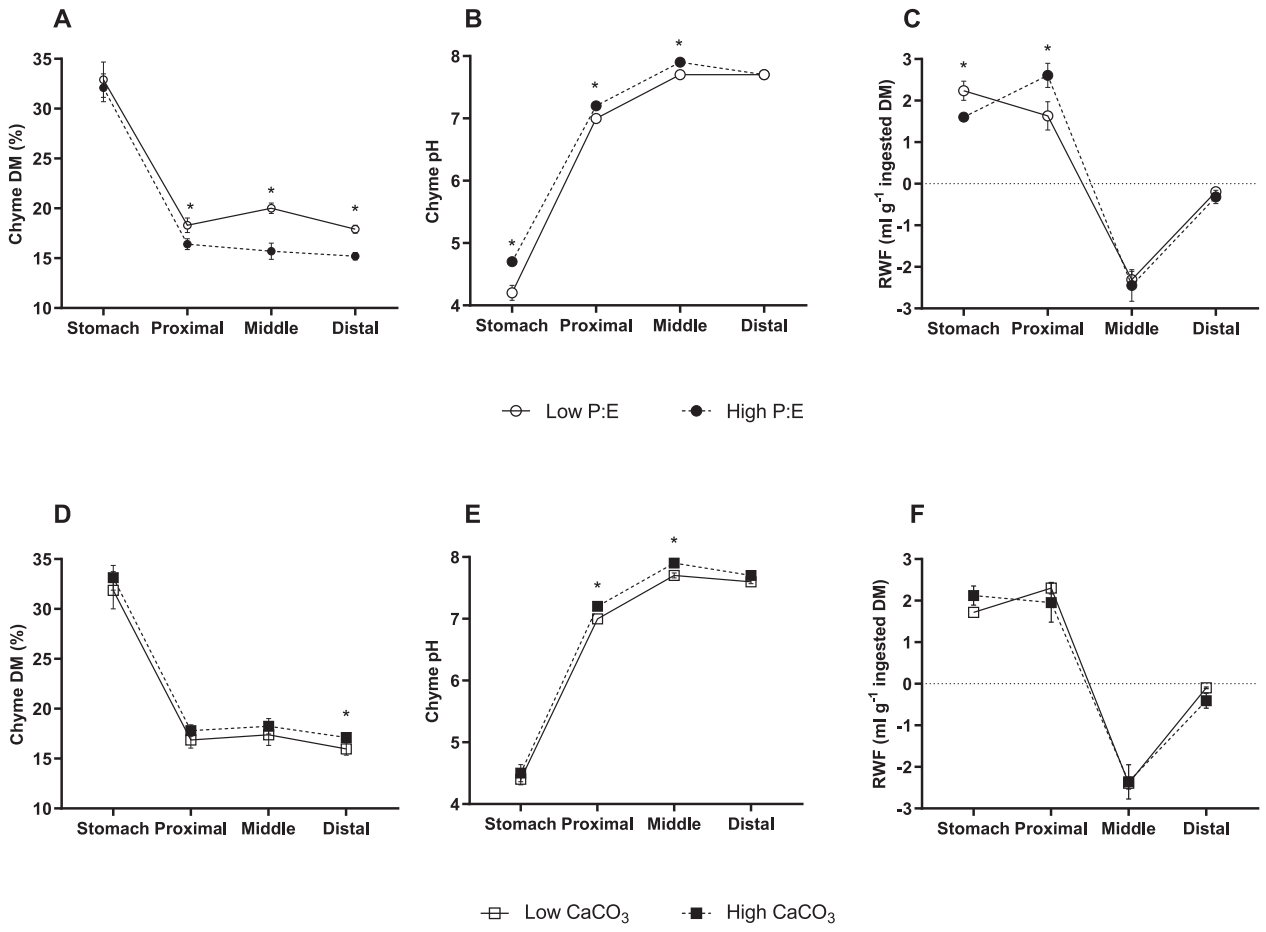

Fig. 1. Chyme dry matter (DM), pH and relative water fluxes as affected by the main effects of dietary protein to energy ratio (P:E) and calcium carbonate levels ($CaCO_3$) in the stomach, proximal, middle and distal intestine. (A) chyme DM, (B) chyme pH and (C) RWF as affected by dietary P:E ratio. (D) chyme DM, (E) chyme pH and (F) RWF as affected by dietary $CaCO_3$. All the analyses were done by a 3-way ANOVA. Main effect of P:E: values per diet are averaged over both $CaCO_3$ levels ($n = 12$). Main effect of $CaCO_3$: Values per diet are averaged over both P:E levels (n = 12). Asterisks (*) indicate significant differences and the error bars are pooled standard error of mean (pSEM). Mean values and level of significance are given in Supplementary table S1.

fish fed the high P:E diet had a lower water influx in the stomach and a higher ($p < 0.05$) water influx in the proximal intestine than fish fed the low P:E diet (Fig. 1C).

3.3. Electrolyte fluxes

Time and interactions effects with time were minimal for electrolyte fluxes (Ca, Na, and K) in the GIT, except for the K flux in the middle intestine. Due to the low number of time effects and the absence of interaction effects with P:E ratio and $CaCO_3$ on electrolyte fluxes, only the dietary effects are depicted in Fig. 2. The full 3-way ANOVA results with all treatments means including sampling time is reported in the Supplementary table S2.

Overall, the electrolyte fluxes were affected by dietary P:E ratio, while no effect of dietary buffering capacity was detected (Fig. 2).

Relative Ca flux (R Ca F) was negative in the stomach (Ca efflux), but it was not affected by the dietary P:E ratio (Fig. 2A). R Ca F was positive in the proximal intestine (Ca influx), and it increased ($p < 0.05$) in fish fed the low P:E diet. Net calcium efflux occurred in the middle intestine, while a minor Ca influx occurred in the distal intestine, but no dietary effect was detected.

Relative Na flux (R Na F) was negative (Na efflux) in all GIT segments except for the proximal intestine (Fig. 2B, E). R Na F was only affected by dietary P:E ratio in the distal intestine (Fig. 2B). In the distal intestine, R Na F was lower ($p < 0.05$) in fish fed the high P:E diet compared to low P:E diet.

Relative K flux (R K F) was negative (K efflux) in all the segments of the GIT, except for the distal intestine (Fig. 2C, F). In the stomach, the negative K flux (efflux) increased ($p < 0.05$) in fish fed the low P:E diet. In the middle intestine, K efflux increased ($p < 0.01$) in fish fed the high P:E diet (Fig. 2C).

3.4. Apparent digestibility

Nutrient apparent digestibility coefficients (ADCs) were affected both dietary factors (Table 3). Except for P ADC, no interaction effect between P:E ratio and $CaCO_3$ was observed for any of the measured ADCs.

ADCs of all macronutrients, energy, Ca and P were affected by dietary P:E ratio. The ADC of organic matter (OM), dry matter (DM), crude protein (CP), fat and energy increased ($p < 0.01$) in fish fed the high P:E diet compared to the low P:E diet. In contrast, fish fed the low P:E diet had a higher ($p < 0.05$) carbohydrate, Ca and P digestibility compared to the high P:E diet.

Except for DM, dietary $CaCO_3$ had no effect on macronutrient digestibility. In contrast, fish fed the high $CaCO_3$ diet had lower ($p < 0.001$) Ca, P and K digestibility compared to the low $CaCO_3$ diet.

3.5. Progression of digestion

Fig. 3 depicts the progressive digestibility values of crude protein (CP ADC) and phosphorus (P ADC) as affected by the contrasting dietary P:E

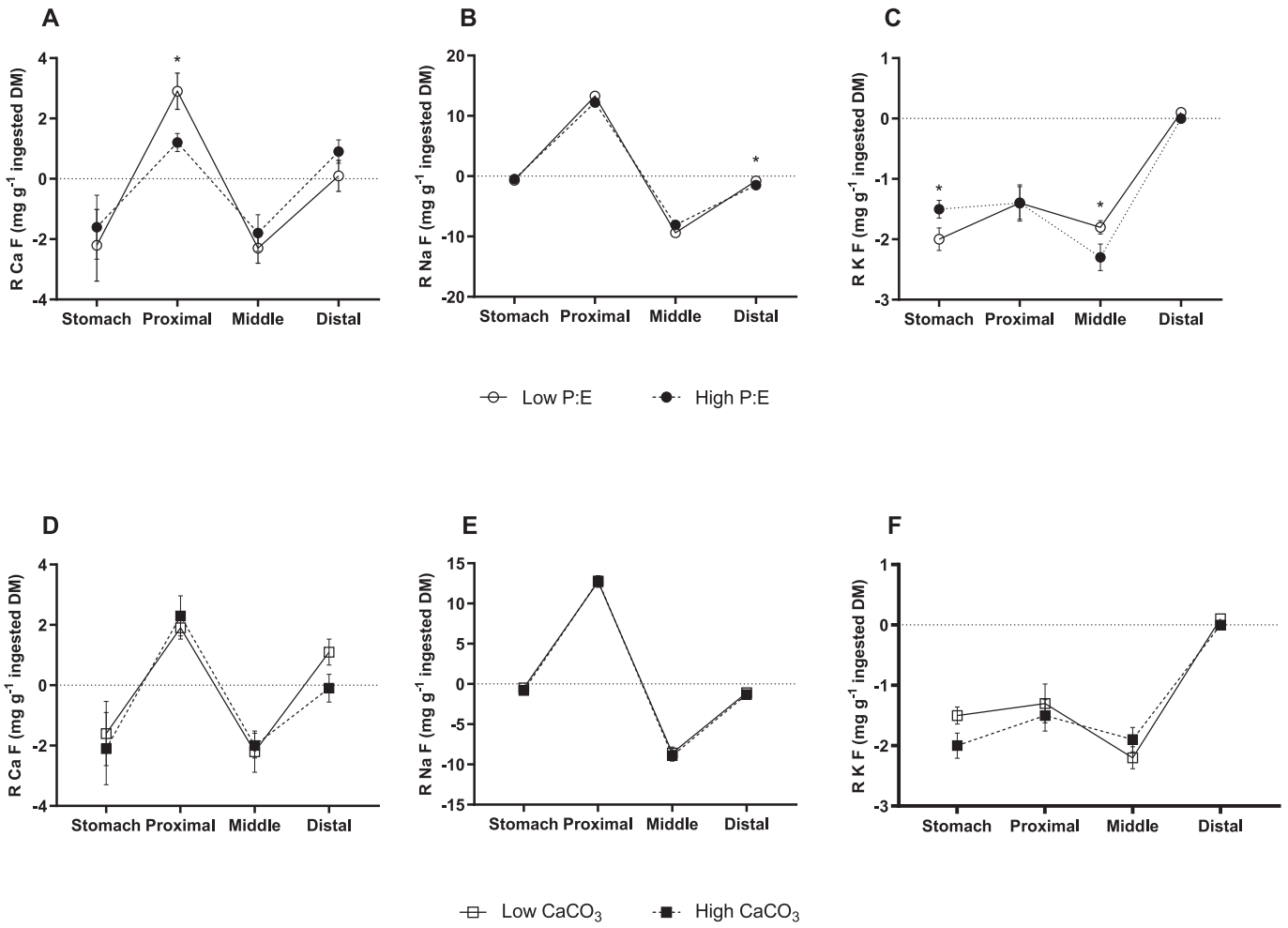

Fig. 2. Relative calcium fluxes (R Ca F), relative sodium fluxes (R Na F) and relative potassium fluxes (R K F) as affected by the main effects of dietary protein to energy ratio (P:E) and calcium carbonate levels ($CaCO_3$) in the stomach, proximal, middle and distal intestine. (A) R Ca F, (B) R Na F and (C) R K F as affected by dietary P:E ratio. (D) R Ca F, (E) R Na F and (F) R K F as affected by dietary $CaCO_3$. All the analyses were done by a 3-way ANOVA. Main effect of P:E: values per diet are averaged over both $CaCO_3$ levels ($n = 12$). Main effect of $CaCO_3$: Values per diet are averaged over both P:E levels (n = 12). Asterisks (*) indicate significant differences and the error bars are pooled standard error of mean (pSEM). Mean values and level of significance are given in Supplementary table S2.

Table 3
Nutrient apparent digestibility coefficients (ADCs) of rainbow trout as affected by the main effects of dietary protein to energy ratio (P:E) and calcium carbonate levels ($CaCO_3$) during week 6 of the experiment.

	Low P:E		High P:E			*p-values*		
ADC (%)	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	pSEM	P:E	$CaCO_3$	P:E*$CaCO_3$
Organic matter	83.8	83.8	89.0	88.5	0.45	***	ns	ns
Dry matter	81.8	79.6	86.3	83.5	0.54	***	***	ns
Crude protein	96.3	96.1	97.3	97.2	0.14	***	ns	ns
Fat	93.9	93.9	95.0	94.4	0.22	**	ns	ns
Carbohydrates	68.8	69.3	67.0	66.0	0.92	**	ns	ns
Energy	86.5	86.5	91.1	91.0	0.38	***	ns	ns
Ash	46.9	32.8	49.0	30.8	1.70	ns	***	ns
Calcium	22.8	20.2	9.2	12.7	1.79	***	ns	ns
Phosphorus	60.6^{d}	49.1^{b}	55.2^{c}	38.7^{a}	0.91	***	***	*
Potassium	97.5	96.5	97.4	96.6	0.16	ns	***	ns

pSEM, pooled standard error of mean; ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. The data were analysed by a 2-way ANOVA using dietary P:E ratio and $CaCO_3$ level as independent factors. Values are means (n = 6) and pooled standard error of the mean (pSEM).

ratio and $CaCO_3$ level. The full 3-way ANOVA output with all treatments means including the effect of sampling time and their interactions are given in Supplementary table S3.

Except for a $CaCO_3$ effect on CP ADC in the proximal intestine ($p < 0.05$), there were no significant main effects of dietary P:E ratio and $CaCO_3$ level on digestion kinetics of CP and P in the GIT (Fig. 3). Numerically, CP ADC kinetic was higher in fish fed a low P:E diet in all GIT segments except the distal intestine (Fig. 3A). In the distal intestine, the higher CP ADC in fish fed the high P:E diet is consistent with faecal digestion (Table 3). For both dietary treatments, P ADC increased from the stomach to the middle intestine and decreased in the distal intestine (Fig. 3B, D).

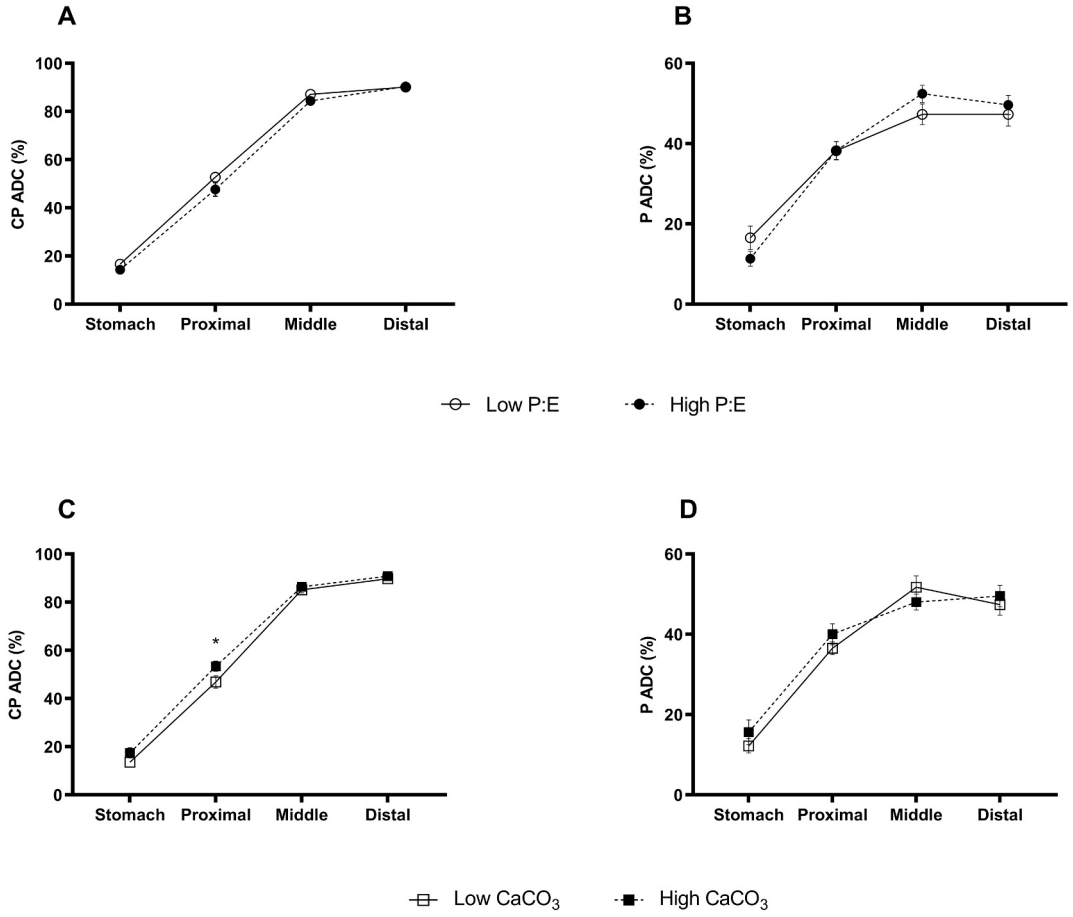

Fig. 3. Progression of digestion of crude protein (CP ADC) and phosphorous (P ADC) as affected by the main effects of dietary protein to energy ratio (P:E) and calcium carbonate levels ($CaCO_3$) in the stomach, proximal, middle and distal intestine. (A) CP ADC, (B) P ADC as affected by dietary P:E ratio. (C) CP ADC, (D) P ADC as affected by dietary $CaCO_3$. All the analyses were done by a 3-way ANOVA. Main effect of P:E: values per diet are averaged over both $CaCO_3$ levels ($n = 12$). Main effect of $CaCO_3$: Values per diet are averaged over both P:E levels (n = 12). Asterisks (*) indicate significant differences and the error bars are the pooled standard error of mean (pSEM). Mean values and level of significance are given in Supplementary table S3.

4. Discussion

According to literature, the buffering capacity of a diet is dependent, among other factors, on the amount of $CaCO_3$ added and on the protein level (Giger-Reverdin et al., 2002; Levic et al., 2005). In the current study, $CaCO_3$ supplemented diets had the highest buffering capacity and highest pH when mixed with water (Table 1). As a result, we hypothesized that the high dietary buffering capacity and pH of $CaCO_3$ supplemented diets would increase chyme pH in the stomach of rainbow trout postprandially, affecting water and electrolyte fluxes in the gastrointestinal tract (GIT) as well as digestion kinetics.

Our results show that dietary $CaCO_3$ supplementation did not affect chyme pH in the stomach of freshwater rainbow trout (*Oncorhynchus mykiss*). Parma et al. (2019) reported a similar finding, observing that including $CaCO_3$ in the diet had no effect on chyme pH in the stomach of European sea bass (*Dicentrarchus labrax*). In contrast, Goodrich et al. (2022a) found that increasing dietary buffering capacity by adding $CaCO_3$ increased stomach chyme pH in juvenile rainbow trout (*Oncorhynchus mykiss*).

In contrast to dietary $CaCO_3$ addition, the P:E ratio had an impact on chyme pH in the stomach and proximal intestine. The P:E effect on stomach chyme pH could be due to the buffering capacity of the diet but also to the contrast between protein and non-protein energy (starch + fat) ratio. The P:E ratio had minor impact on diet pH but had an effect on dietary BC, although it was much smaller compared to that of $CaCO_3$ supplementation (Table 1). However, because the BC of the high P:E diet was not as high as that of the $CaCO_3$ supplemented diets, we could speculate that the diet effect on chyme pH is caused by the dietary macronutrient composition rather than its BC. Previous research found no differences in stomach and intestinal chyme pH when the macronutrient ratio in the diet was changed, specifically when protein was kept constant and the ratio between fat and starch was changed (Harter et al., 2013). In current study, however, where the type of non-protein energy was kept similar (i.e., starch-to-fat ratio), exchanging protein for non-protein energy decreased chyme pH in the GIT of rainbow trout (Fig. 1B). In other words, lowering the dietary protein content led to a lower chyme pH. The contrast in P:E ratio also had an impact on water influx in the stomach, being higher in fish fed low P:E diet. Therefore, the lower chyme pH in the stomach of fish fed the low P:E diet might also be a direct consequence of the increased water influx, suggesting that P:E ratio also had an impact on the amount of acidic secretions added to the stomach. As a result, we could speculate that change in macronutrient composition of a diet affects the stomach chyme pH more than $CaCO_3$ supplementation.

Previous research has shown that water influx in the stomach promotes protein denaturation (Ciavoni et al., 2023; Elesho et al., 2022; Harter et al., 2015; Saravanan et al., 2013). However, in the current study, no significant effect on protein digestion kinetic was found in the stomach of fish fed the low P:E diet (Fig. 3A). One possible explanation is that the low P:E diet contains more starch, which is known to increase water addition to the stomach (Elesho et al., 2022; Harter et al., 2015) but also to increase chyme viscosity (Amirkolaie et al., 2006). According

to Tran-Tu et al. (2019), higher chyme viscosity in the stomach delay the passage rate of chyme from the stomach to the proximal intestine and reduces CP digestibility. Furthermore, Harter et al. (2015) and Elesho et al. (2022) reported that when dietary starch was replaced with fat, chyme DM levels in the stomach of African catfish increased. In fact, it is well known that fats slow gastric emptying more than proteins and carbohydrates (Burn-Murdoch et al., 1978; Hunt and Stubbs, 1975). In this study, however, we did not find an increase in chyme DM in the stomach of fish fed the low P:E diet. In contrast, fish fed the low P:E diet had a higher chyme DM and a lower relative water influx in the proximal intestine. Previous research found that higher water influx occurs in the stomach of fish fed a starch-based diet and higher water influx occurs in the proximal intestine of fish fed a high-fat diet (Harter et al., 2013). This could be because starch hydrolysis in the stomach produces a large amount of mono- and disaccharides with a higher water binding capacity, whereas in the proximal intestine, the high-fat requires more bile acid addition for lipid denaturation (Anguita et al., 2006). In the current study, where fat-to-starch ratio is kept constant, we found that also contrasting protein levels in the diet can affect water influx in the stomach and proximal intestine. In previous research, replacing fat with starch resulted in stronger alterations in water fluxes in the GIT as well as DM content (Elesho et al., 2022; Harter et al., 2015). In this study, the contrast in dietary P:E ratio had a minimal effect on water fluxes in the intestine, but the effect on chyme DM was significant. However, the large differences in DM levels in the intestine are not reflected in digestion kinetics. The lower DM content in the intestine of fish fed the high P:E diet did not lead to a significant alteration in the kinetic of digestion. Although the digestion kinetics of CP was slightly lower in fish fed the high P:E diet, this was reversed in the faecal digestibility (ADC). Previous research has shown that when the protein level in the diet increases, the endogenous protein loss is lower resulting in increased protein ADC (Fountoulaki et al., 2005), which could explain the outcome of the present study. The contrast in P:E ratio also affected faecal Ca digestibility. The protein mixture used in the current study in the diet formulation was fully plant-based ingredients. Therefore, the lower faecal Ca ADC in fish fed the high P:E diets could be attributed to a difference in the phytate content between both P:E ratios, as phytate is known to reduce mineral bioavailability (Francis et al., 2001).

Digestive fluids, such as gastric, gall bladder and pancreatic secretions are the main sources of ion influxes into the GIT (Grosell, 2006). Several studies on rainbow trout have shown that ion fluxes are altered after the consumption of a meal (Bucking et al., 2011; Bucking and Wood, 2006; Ciavoni et al., 2023). The electrolytes fluxes measured in the current study (Fig. 2) show that the main dietary factor responsible for the differences in the GIT is the P:E ratio. In the proximal intestine, fish fed a high P:E diet had a lower Ca influx but a higher water influx. This suggests that the feed composition, in addition to calcium-rich bile acid and pancreatic secretions, promotes water influx (endogenous or exogenous origin). Therefore, together with water fluxes, electrolyte fluxes can be altered when the macronutrient composition in the diet is changed. The trend of Na and K fluxes in the GIT reflect those measured in freshwater rainbow trout in previous studies (Bucking and Wood, 2006; Ciavoni et al., 2023). Ciavoni et al. (2023) investigated the influence of dietary electrolyte balance (dEB) on Na and K fluxes. They found that the dietary dEB only affected Na fluxes in the stomach and had no effect on K flux. However, the potential effect of macronutrient composition on electrolyte fluxes was not considered in those studies. In the current study, changing the P:E ratio had an effect on ion fluxes in different gut segments, while dietary $CaCO_3$ supplementation had no effect. This suggests that macronutrient composition, rather than buffering capacity, is the primary factor influencing electrolyte fluxes in the GIT in freshwater rainbow trout. However, also in this case, it is difficult to distinguish between a starch or a fat effect.

Overall, the results of this study show that dietary P:E ratio has an effect on chyme conditions and water fluxes in the gastrointestinal tract of freshwater rainbow trout, whereas dietary $CaCO_3$ has almost no effect. In previous research, $CaCO_3$ has been used as a strong feed buffer additive (Goodrich et al., 2022a). However, our findings suggest that differences in chyme characteristics, water and electrolyte fluxes are caused primarily by dietary macronutrient composition rather than dietary BC. Furthermore, both dietary factors had a significant impact on faecal digestibility but not on kinetics of digestion, implying that fish can compensate for changes in chyme condition along the GIT caused by dietary factors.

CRediT authorship contribution statement

Elisa Ciavoni: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Marit Nederlof:** Methodology, Validation, Formal analysis, Supervision. **Jaimy Rooijakkers:** Methodology, Formal analysis, Investigation. **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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739674.

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