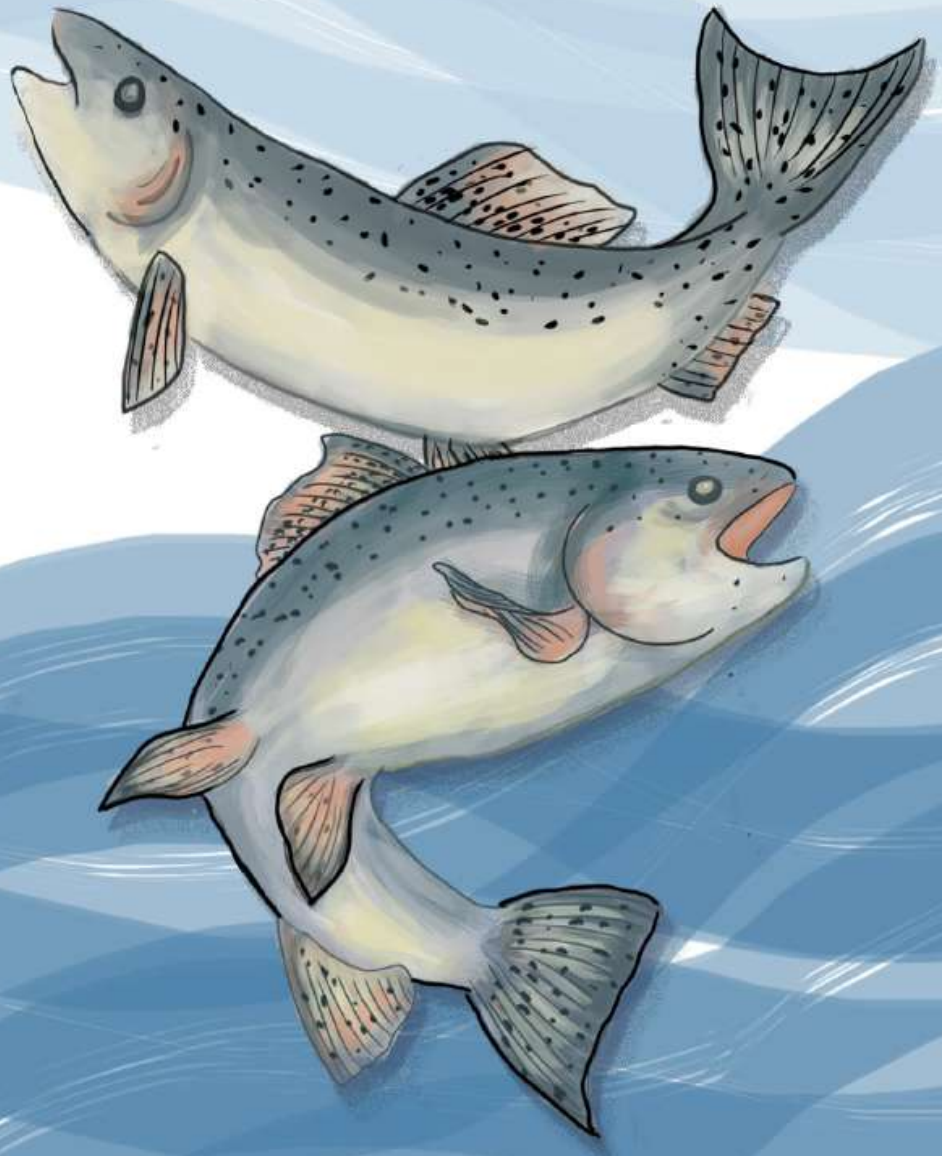
The Interplay of Digestion and Osmoregulation in the Gastrointestinal Tract of Salmonids

Elisa Ciavoni



Propositions

1. Dietary factors affect osmoregulation in the gut of salmonids differently depending on water salinity.
   (this thesis)

2. In seawater conditions, ingested water moves directly to the proximal intestine, bypassing the stomach.
   (this thesis)

3. Artificial intelligence should be used in academic writing, as long as supervised by humans.

4. As global change accelerates, the rapid evolution of parasites will outpace the ability of hosts to adapt.

5. For a gender equal society, more women should be employed in higher job positions.

6. To reduce socioeconomic inequalities, Western African countries should refrain from signing fishing agreements with foreign governments.

Propositions belonging to the thesis, entitled

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Elisa Ciavoni
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Elisa Ciavoni

Thesis Committee

Promotor
Dr Johan W. Schrama
Associate Professor, Aquaculture and Fisheries Group
Wageningen University & Research

Co-Promotors
Dr Antony J. Prabhu Philip
Senior Scientist, Feed and Nutrition Research group
Institute of Marine Research, Bergen, Norway

Dr Øystein Sæle,
Senior Scientist, Feed and Nutrition Research group
Institute of Marine Research, Bergen, Norway

Other Members
Prof. Dr M.J. Barbosa, Wageningen University & Research
Dr V.F. Mota Norwegian University of Life Sciences, Norway
Dr S. Subramanian, Selko, Nutreco, Norway
Dr S. de Vries, Wageningen University & Research

This research was conducted under auspices of the Graduate School
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The Interplay of Digestion and Osmoregulation in the Gastrointestinal Tract of Salmonids

Elisa Ciavoni

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CHAPTER 1

General introduction

1.1 Growth in aquaculture

Aquaculture is the world's fastest growing industry, contributing to food security and nutrition for humans (FAO, 2022). With persisting pressure on wild fish stocks, aquaculture is the only viable option for meeting global demand for marine proteins for human consumption. Aquaculture production continues to rise, accounting for 49% of total aquatic production (178 million tons) in 2020 and is expected to grow up to 53% by 2030, according to the state of world fisheries and aquaculture (FAO, 2022). However, about 16 million tons of aquatic organisms were captured to produce fish meal and fish oil in 2020 (FAO, 2022). To date, as aquaculture production increases, so does demand for fish meal and fish oil to produce aquafeeds, particularly for carnivorous fish species. Fishmeal and fish oil are highly valued as easily digestible components of farmed fish feed, providing essential nutrients such as proteins, essential amino acids, lipids, and energy (FAO, 2020). To meet their rigorous nutritional requirements, high-value species such as shrimp and salmon require elevated levels of marine ingredients, making shrimp and salmon aquaculture the primary consumers of fishmeal and fish oil (Froehlich et al., 2018; Naylor et al., 2009; Shepherd et al., 2017; Tacon and Metian, 2015). While omnivorous species need less fishmeal in their feed, their large production volumes place significant demands on global fishmeal resources (Tacon and Metian, 2008). Market dynamics, including pricing, availability, and regulations, have been driving a shift toward substituting marine ingredients with plant-based, animal-derived byproducts, and innovative feed components (Davies et al., 2019; Froehlich et al., 2018; Gatlin et al., 2007; Pelletier et al., 2018; Shepherd and Bachis, 2014). This transition, combined with the expanding aquaculture sector, could potentially exert additional pressure on essential agricultural resources, resulting in socio-economic and environmental repercussions (Blanchard et al., 2017; Froehlich et al., 2018; Malcorps et al., 2019; Roberts, 2015). Despite the modest land use in aquaculture, accounting for approximately 4% of the global animal feed supply (Troell et al., 2014), concerns have long persisted about its impact on

marine fish stocks and the continued supply of marine ingredients, primarily fishmeal and fish oil (Blanchard et al., 2017; Naylor et al., 2000; Stead, 2019; Troell et al., 2014). Currently, the main alternative ingredients used in feed formulation come from crops (soybeans, wheat, corn, or rice) and byproducts recovered from livestock and poultry processing (Glencross et al., 2007). Crop and plant oil production is far greater than fish meal and fish oil production on a global scale. In 2020, global primary crop production was 9.3 billion tons, with cereals accounting for most crops produced (30%), followed by sugar crops (23%), vegetables, and oil crops (12% each) (FAO, 2022). The vast majority of crop and plant oil production is consumed directly by humans. However, more than 30% of cereal crop production is used in animal feeds, including aquafeeds (FAO, 2022). Between 1990 and 2020, the use of terrestrial plant ingredients as protein sources for carnivorous fish species in aquaculture feed formulation increased significantly (Napier et al., 2020). For example, in 2020, feed produced for Norwegian salmon farming used 22.4% and 73.1% of ingredients derived from marine and plant-based ingredients, respectively (Aas et al., 2022; Figure 1). However, commodity prices for plant-based ingredients have risen dramatically since 2007, due to increased demand for their use for human food, animal feed, and biofuel production (Hardy, 2010). A large portion of the resources used in livestock and aquaculture feeds can directly be consumed by humans (FAO, 2022). As a result, the cost of plant-based feed production is rising as it competes with direct human consumption (Sandström et al., 2022). Furthermore, the confluence of several factors, including climate change, COVID-19 (in 2020), increasing fuel prices, and wars (e.g., Russia-Ukraine in 2014 and 2022; Yemen in 2014), have impaired the global availability and supply of several raw materials for feed production, severely affecting supplies and pricing (Ahmed and Azra, 2022).

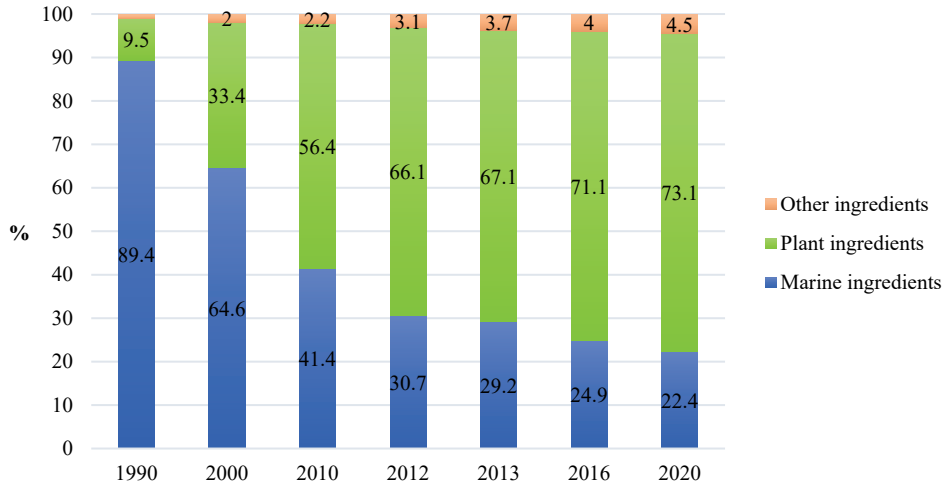

Figure 1. Feed ingredient sources (% of feed) in Norwegian salmon feed in 2020 compared to previous years (adapted from Aas et al., 2022). Marine ingredients: marine proteins, marine oils. Plant ingredients: plant proteins, plant oils, carbohydrates. Other ingredients: micronutrients (i.e., vitamin and mineral premixes, astaxanthin, and crystalline amino acids), insect meal, single-cell protein, fermented products, and microalgae.

Another challenge with plant-based ingredients is their composition (i.e., macronutrients, micronutrients, anti-nutritional factors, amino acids, and mineral profile) (NRC, 2011). The amino acid (AA) composition of plant-based proteins does not reflect fish nutritional requirements, as they are often deficient in one or more essential AA (e.g., lysine and methionine) (Elesho et al., 2021). As a consequence, when using plant protein sources, aquafeeds need supplementation of synthetic amino acids (Hardy, 2010). Furthermore, plant protein sources contain a variety of antinutritional factors such as phytic acid, saponins, tannins, soluble non-starch polysaccharides, and gossypol that are not or partially removed through processing or pelleting (Francis et al., 2001b; Hardy, 2010).

In general, future fish diets will include a broader range of alternative ingredients as substitutes for fish meal and fish oil. As a result, nutritional research and feed development for aquaculture species rely heavily on feed ingredient evaluation (Glencross et al., 2007). Feed ingredient evaluation

should consider several factors such as digestibility, palatability, and nutrient availability, as well as the anatomy, digestive physiology, environment, and trophic level of the fish. The ability of fish to digest food is critical for aquaculture success because it determines how well nutrients in the diet are used for energy production and growth (Harter et al., 2015). Furthermore, undigested nutrients may have an environmental impact, emphasizing the need for more digestible ingredients (Wilfart et al., 2023). Along with digestion, the gastrointestinal tract (GIT) in fish is involved in osmoregulation, immune functions, and acid-base balance.

The following paragraphs provide a more in-depth understanding of the role of the GIT in digestion, osmoregulation, and acid-base balance. The stomach and intestine will be the primary focus of this thesis. Furthermore, the current state of the art on the interaction of feeding and osmoregulation is presented. The information in this section is intended to supplement and support the experimental chapters.

1.2 Gastrointestinal tract: structure and digestion

1.2.1 Structure

In fish, the digestive tract is divided into four sections: the headgut, foregut, midgut, and hindgut (Wilson and Castro, 2010). The headgut, which includes the mouth and pharynx, is in charge of acquiring and, in some species, mechanically processing food. Physical breakdown of prey animals begins in the mouth cavity of some fish. The great white shark (*Carcharodon carcharias*) and other sharks chop their prey with their teeth and jaws before swallowing it. Other carnivorous fish such as salmonids (e.g., rainbow trout, Atlantic salmon) and bottom-feeding fish species (e.g., flounder, eels, cod, catfish etc.), on the other hand, swallow their food directly into the stomach (Wilson and Castro, 2010). The foregut, which includes the esophagus and stomach, is the first section where the chemical digestion of food begins. Across fish species, the stomach is the most diverse part of the gut. However,

about 20% of total teleosts species have evolutionary lost the (functional) stomach (Wilson and Castro, 2010). When present, the stomach shape can be straight, as in some freshwater and seawater species of goby fish; Y (or caecal shaped), typically from predatory fish of large amount of preys such as eels or mackerel; and U (or J or siphonal shaped), commonly found in carnivorous fish species with a large meal size such as sharks, rays and salmonids (Bucking and Anderson, 2020; Egerton et al., 2018). The stomach is the site for storage and the initial mechanical and enzymatic breakdown of the ingested diet. The anterior cardiac region of the fish stomach typically lacks secretory functions, whereas the central and distal regions contain gastric glands (Anderson, 1986; Ostrander, 2000). These gastric glands are lined with goblet cells that synthetize and secrete mucus into the lumen, which is a chemical and physical barrier (Morrison and Wright, 1999; Cain and Swain, 2011), and oxynticopeptic cells that secrete hydrochloric acid (HCl) and pepsinogen (Bakke et al., 2010). The former is secreted by apical proton pumps H^+/K^+-ATPases, while the latter by basal zymogen granules of oxynticopeptic cells (Yúfera et al., 2012). Secreted HCl lowers stomach pH and converts the inactive pepsinogen into the active proteolytic enzyme pepsin, which breaks down proteins into peptides (Wu et al., 2009; Yúfera et al., 2004). The pH of the stomach can range between 1 and 5, depending on the species, developmental stage, feeding state, and time after a meal (Bakke et al., 2010). However, the optimal acidity for pepsin activity is suggested to be between pH 1.5 and 3 (Rønnestad et al., 2013; Walford and Lam, 1993; Yúfera et al., 2012). The midgut, or intestine, is where nutrient hydrolysis continues and most absorption occurs. The primary function of the intestine is to complete the digestive processes begun in the stomach and to facilitate nutrient absorption. The epithelium of the intestine is composed of a single layer of absorbent cells, enterocytes, with an apical microvilli brush border. The surface area of the intestinal epithelium is increased by folding (primary, secondary, and/or tertiary), as well as lengthening the intestine through convolution in teleost fish species (Wilson and Castro, 2010). In carnivorous

fish species, the anterior part of the intestine is frequently associated with pyloric ceca, which are blind-ended ducts. The pyloric ceca further increase the surface area available for digestion and absorption. Goblet-type mucous cells, and enterocytes are dispersed throughout the epithelium. Intestinal enterocytes express H^+-ATPase and Na^+/H^+ exchanger basolaterally and apically, as well as an apical expression of Na^+/H^+ exchanger and Na^+/HCO_3^- cotransporter, which is important for facilitating transepithelial nutrient uptake and ion regulation (Wood et al., 2019). The hindgut, or rectum, is the last section of the digestive tract and ends with a muscular sphincter that empties into the anus (Wilson and Castro, 2010).
Throughout the whole thesis, the segments of the GIT are referred as stomach, proximal, middle, and distal intestine.

1.2.2 Digestion

The stomach of a fish is where the initial physical and enzymatic breakdown of food takes place. Secreted HCl aid in activating the proteolytic enzyme pepsin, which leads to protein hydrolysis (Wu et al., 2009; Yúfera et al., 2004). The resulting mixture of food and secretions, known as chyme, is then transferred into the intestine via the pyloric sphincter (Bakke et al., 2010). Once in the intestine, digestive enzymes (like lipases, amylases, and proteolytic enzymes trypsin/chymotrypsin) are secreted into the lumen of the pyloric ceca and/or proximal intestine via pancreatic and bile ducts (Kurokawa and Suzuki, 1995; Morrison et al., 2004). Furthermore, intestinal secretions contain electrolytes, most notably bicarbonate, which is needed to neutralize the acidic pH of chyme coming from the stomach and promote enzyme activity. The optimal pH for enzyme activation in the intestine of fish is between 7 and 9 (Deguara et al., 2003; Fard et al., 2007). Following nutrient hydrolysis and solubilization of ingested nutrients into molecules and elements suitable for transport across the intestinal wall, the resulting smaller fragments are further digested at the intestinal epithelium by enzymes located in the brush border membrane of the enterocytes, releasing molecules small

enough for absorption, namely small peptides and amino acids, and monosaccharides (Bakke et al., 2010).

1.3 Gastrointestinal tract: acid-base balance

Acid-base balance in fish is influenced by environmental factors such as water dissolved oxygen (O_2) and carbon dioxide (CO_2), salinity, and pH. The gills, kidneys and the gastrointestinal tract are the primary organs involved in regulating acid-base balance in fish (Bucking et al., 2010). The flow of water across the gills provides oxygen to support aerobic metabolism. CO_2 produced by fish metabolism is transported as HCO_3^- in the plasma by the apical Cl^-/HCO_3^- exchanger at the gill cell level in teleost fish (Gilmour and MacNeill, 2003; Wood, 2019). The accumulation of HCO_3^- in the plasma is directly related to the acid-base balance of the gastrointestinal tract during digestion.

During digestion, the rate of acid secretion (HCl) by the oxynticopeptic cells in the stomach increases. As a result, the pH of the stomach drops. Because of the low gastric pH, all free HCO_3^- will be converted into CO_2 via the reversible hydration/dehydration reaction: $H^+ + HCO_3^- \leftrightarrow CO_2 + H_2O$. The enzyme carbonic anhydrase (CA) catalyzes this reaction (Evans et al., 2005; Gilmour and Perry, 2009; Marshall and Grosell, 2005). CO_2 then diffuses into the stomach epithelia, where it is rehydrated by CA and excreted into the bloodstream as HCO_3^- (Wood et al., 2019). The accumulation of HCO_3^- in the bloodstream after feeding causes a rise in blood pH. This phenomenon is known as post-prandial alkaline tide (Wood et al., 2019). As previously stated, fish can lower blood alkalinity by releasing HCO_3^- into the water as dissolved CO_2 through the gills (Evans et al., 2005).

The acid-base balance (Na^+, K^+, Cl^-) can be challenged when farmed fish consume feed. Dietary factors, particularly mineral composition, can affect the electrolyte balance (dEB) and buffering capacity (BC) of the diet, causing further acid-base imbalance during digestion. dEB indicates the balance between monovalent cations (Na^+ and K^+) and anions (Cl^-) in the feed, with

lower dEB values indicating acidic diets and higher values indicating alkaline diets (Saravanan et al., 2013b; Sauvant et al., 2004). Changes in dEB can alter stomach pH and promote compensatory mechanisms such as acid-base secretion or excretion, resulting in temporary metabolic alkalosis or acidosis following feeding (Bucking and Wood, 2008; Levic et al., 2005; Magnoni et al., 2018a). Dietary BC is defined as the ability of the feed to contrast a pH change after adding an acidic or basic solution (Giger-Reverdin et al., 2002). Feeds with a high mineral concentration have a higher BC. Plant ingredients, on average, have lower BC than fish meal (Giger-Reverdin et al., 2001; Parma et al., 2019). Similarly to dEB, changes in dietary BC may alter the acid-base balance and osmoregulation in the gastrointestinal tract of fish during digestion (Goodrich et al., 2022a).

1.4 Gastrointestinal tract: osmoregulation

Depending on their living environment and/or migratory life cycle, some fish species are required to make transitions between different osmoregulatory strategies. Osmoregulation is a physiological mechanism aiding in maintaining salt and water balance (homeostasis) across body membranes. Fish use different strategies to regulate the osmolality of their body fluids (plasma, lymph, and interstitial fluids) depending on water salinity (osmoconformity), at a higher level in a dilute environment or freshwater (hyperosmoregulation), and at a lower level in a concentrated environment or saltwater (hypo-osmoregulation) (Marshall and Grosell, 2005; Figure 2). Only a small number of fish species can live in both freshwater and saltwater ecosystems (euryhaline). The epithelia of fish gastrointestinal tract, gills, kidneys, and skin play a role in the passive and active movement of ions and water (osmoregulation). Freshwater (FW) fish lose ions (Na^+, Cl^-, K^+) via diffusion from the gills and actively absorb ions across branchial and gastrointestinal epithelia to counteract diffusive losses. Furthermore, a significant amount of water (~ 1 mOsm) is excreted through highly diluted

urine, highlighting the role of kidneys in osmoregulation in freshwater fish (Evans and Claiborne, 2008; Grosell, 2010; Marshall and Grosell, 2005).

Seawater (SW) fish actively ingest water (~ 1000 mOsm) and excrete ions to maintain osmotic balance. Ingested seawater is initially desalinated in the esophagus, lowering its osmolality to around 500 mOsm, resulting in a fluid that closely matches the osmolality of plasma (Grosell and Genz, 2006). Desalinated seawater from the esophagus moves to the stomach, where it may be further desalinated before entering the anterior intestine at around 400 mOsm osmolality (Evans and Claiborne, 2008). In seawater, to counteract water loss and dehydration, Na^+ and Cl^+ absorption occurs in the intestine and kidneys, and water follows by osmosis (Breves et al., 2020; Takvam et al., 2021). Ion uptake in the intestine promotes compensatory active ion excretion via the gills (Na^+, Cl^-, and K^+) and the anus and urinary opening (Ca^{2+}, Mg^{2+}, and SO_4^{2-}) (Hirano and Mayer-Gostant, 1976; Kaneko et al., 2008; Marshall and Grosell, 2005; Takei et al., 2017). In both seawater and freshwater fish, the intestine absorbs Na^+ and Cl^- at comparable rates (Hirano and Mayer-Gostant, 1976).

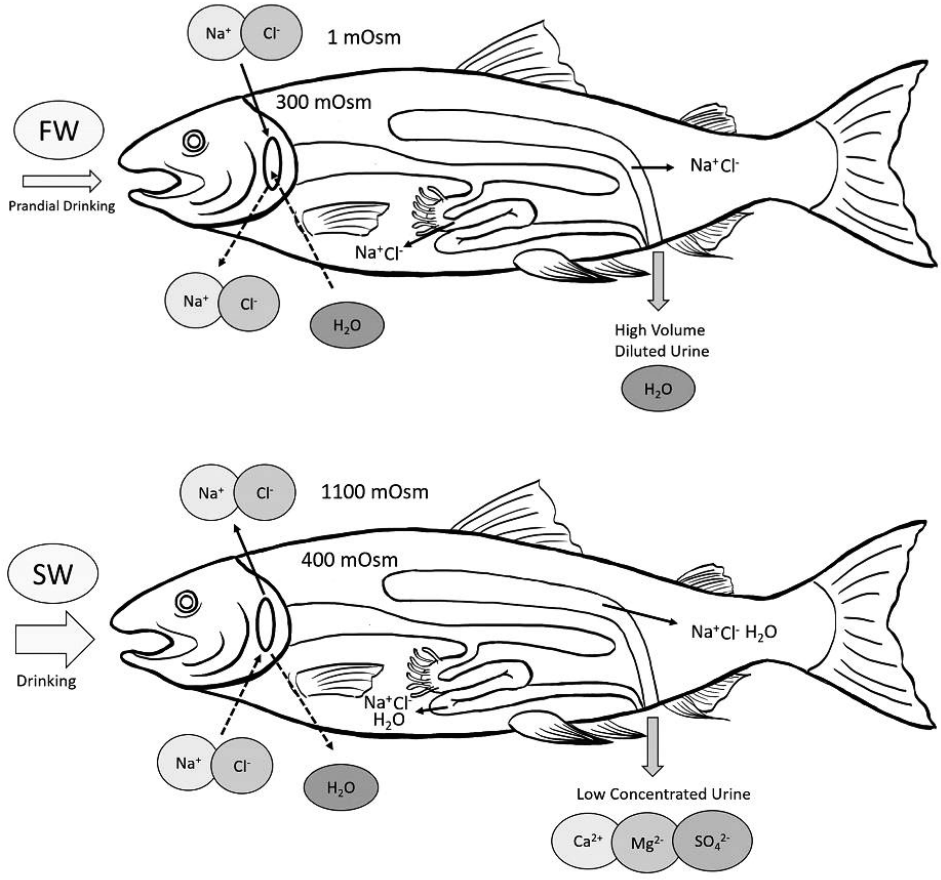

Figure 2. Osmoregulation mechanisms (simplified) in freshwater and seawater-adapted fish. Dashed arrows are passive flows, while solid arrows are active flows. Derived from Evans and Claiborne (2008).

In marine teleosts, the acidic chyme that enters the intestine is neutralized by bicarbonate secretion (HCO_3^-), which derives from the intestinal enterocytes, pancreatic secretions, and bile. As a result, the presence of base concentration tends to rise in the intestinal lumen, causing calcium (Ca^{2+}) and magnesium (Mg^{2+}) carbonate formation and precipitation (Grosell, 2019). The formation and excretion of these aggregates reduce the osmolality of the luminal fluid and enhance water absorption in the intestine (Evans et al., 2013). The biliary HCO_3^- secretion into the intestinal lumen is very low in freshwater fish, and the potential contribution of pancreatic secretions is unknown. However,

rather than being associated with osmoregulatory requirements due to increasing drinking rate in seawater fish, HCO_3^- secretion in freshwater fish is thought to be primarily associated with feeding (Taylor et al., 2007; Wood and Eom, 2019).

The GIT is responsible for many functions in fish, including digestion, osmoregulation, and acid-base balance. Furthermore, it is widely recognized that the intestine regulates water and ion fluxes in both FW and SW fish (Sundell and Sundh, 2012). However, little is known about the interaction of feeding and water salinity on osmoregulation in the gastrointestinal tract of fish.

1.5 Feeding and osmoregulation in the GIT

During feeding, water and ion fluxes as well as acid-base balance in the GIT of fish can be altered (Bucking and Wood, 2006a, 2006b). Previous studies on osmoregulation mainly focused on gastrointestinal tract physiology in non-fed fish (Fuentes et al., 1996a, 1996b; Fuentes and Eddy, 1997; Perrott et al., 1992; Tytler et al., 1990). Thereafter, more studies pointed out that, during feeding, osmoregulation, and acid-base balance in the GIT of fish can be disrupted (Bucking et al., 2011; Bucking and Wood, 2009, 2007, 2006b, 2006a; Eddy, 2007; Taylor and Grosell, 2006; Wood and Bucking, 2010). While extensive research has been done on osmoregulation in fish, there is still a gap in understanding how food consumption and environmental factors such as water salinity influence osmoregulatory processes inside the GIT. This lack of knowledge limits our ability to fully comprehend the complex interplay of feeding, osmoregulation, and the external environment (i.e., water salinity) in fish. Furthermore, as aquaculture feed formulations are increasingly more complex with new ingredients being incorporated as the competition for resources grow, understanding the interaction between dietary composition and osmoregulation in the GIT is critical. This is because different dietary factors may challenge acid-base balance and water fluxes in the GIT differently, impacting digestive processes and overall fish growth.

To date, only few studies investigated the effect of specific dietary characteristics (e.g., macro- and micronutrients content) on chyme conditions, water, and ion fluxes in the GIT of fish (Elesho et al., 2022; Goodrich et al., 2022; Harter et al., 2015, 2013; Magnoni et al., 2018, 2017; Saravanan et al., 2013). Nonetheless, previous research on the interaction between dietary factors and osmoregulation in the GIT, while informative, has primarily been done in freshwater conditions. Therefore knowledge on the interplay between water salinity (osmoregulation) and dietary characteristics (digestion) along the GIT of fish is lacking. In fact, while it is well known that 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), no data exists on the effect of diet composition on water fluxes in the GIT in seawater fish. As a result, to gain a deeper understanding of the interactions between diet composition, osmoregulation, and water salinity, additional research is required to fill the existing knowledge gap.

1.6 Thesis aim and outline

The primary goal of this thesis was to understand the interplay between digestion and osmoregulation function of the gastrointestinal tract and investigate how this interaction is influenced by dietary factors, as well as environmental factors such as water salinity.

Chapters 2 and **3** focused on rainbow trout (*Oncorhynchus mykiss*) under freshwater condition. The effect of dietary electrolyte balance (dEB) was examined in **Chapter 2**, and the impact of dietary macronutrient ratio and buffering capacity (BC) was investigated in **Chapter 3**.

Chapters 4 and **5** focused on Atlantic salmon (*Salmo salar*) under freshwater and seawater conditions. In **Chapter 4**, a dose response trial with increasing water salinity was conducted, and Atlantic salmon were fed a commercial diet. In **Chapter 5**, Atlantic salmon were reared in freshwater and seawater and fed diets with contrasting dEB. It was hypothesized that, depending on water salinity, dEB would affect osmoregulation in the GIT differently.

The General Discussion (**Chapter 6**) of my thesis presents a comprehensive overview of the findings, comparing them with each other and existing literature to provide a broader perspective on their implications.

CHAPTER 2

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

This chapter has been published as:

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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^{-1}) is altered. To better understand the interaction between feeding and osmoregulation along the GIT, two diets with low (-100 mEq kg^{-1}) and high (+600 mEq kg^{-1}) 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 hours) 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 calcium (Ca^{2+}) digestibility, 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 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.

2.1. 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 change 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 milliequivalents per kilogram (mEq kg^{-1}) 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^{-1} compared to a dEB for fishmeal of 253 mEq kg^{-1}. dEB in commercial fish feeds generally ranges between 100 and 300 mEq kg^{-1} (Philip et al., 2022; Tacon and De Silva, 1983). 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 (Fuentes et al., 1996b, 1996a; Fuentes and Eddy, 1997; Perrott et al., 1992; Tytler et al., 1990).

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 (2006a) 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 (Kristiansen and Rankin, 2001; Ruohonen et al., 1997). 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 (Magnoni et al., 2018a; Saravanan et al., 2013b).

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 extend 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.

2.2 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.

2.2.1 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 versus 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 hours after feeding. For parameters measured on chyme, each treatment was tested in triplicates. Final sampling is described later in more detail.

2.2.2 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 was 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^{-1}; pH, 7 ± 0.1; temperature, 14 ± 0.3 °C and conductivity, 3 ± 0.2 mS cm^{-1}. 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^{-1}, 0.2 mg l^{-1}, and 500 mg l^{-1}, respectively.

2.2.3 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^{-1} DM) and 2.1% of $NaCO_3$ to create the high dEB diet (+600 mEq kg^{-1} DM).

Because $CaCl_2$ increases the calcium content of the low dEB diet, 1.3% of $CaCO_3$ was added to the high dEB diet to maintain equal calcium content between diets. 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 grams 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).

2.2.3 Feeding

Fish were hand fed twice a day at 9:00 and 15:30 for 1 hour maximum and feeding level was fixed at 1.5% of body weight day^{-1}. The amount of feed given was equal per fish and the amount of DM was equal per tank 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 minutes 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 to each feeding. In case of mortality, daily feeding level was adjusted based on the remaining number of fish in the respective tank(s).

2.2.4 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 3 or 7 hours 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 weigh and the total wet weight of the chyme (%).

Table 1. Ingredients and nutrient composition of 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^{-1} DM)		
Dry matter (g kg^{-1})	925	955
Crude protein	444	465
Crude fat	182	174
Starch[2]	186	185
Non starch polysaccharides	87	83
Crude ash	89	89
Phosphorus	10.5	10.5
Calcium	14.8	14.7
dEB (mEq kg^{-1} DM)	-98	+600
Diet pH	5.4	7.4

dEB, dietary electrolyte balance. [1]Vitamin mineral premix: Vitamins (IU or mg kg^{-1} 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^{-1} 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 $CaIO_3 6H_2O$), 2 mg. [2]Starch analyses included the free sugar fraction.

2.2.5 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

hours until constant weight. Ash content was determined by incineration in a muffle furnace for 4 hours 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^{2+}, Na^{+} and K^{+} were analysed using inductively coupled plasma mass spectrometry according to the standard NEN 15510 (ICP-MS, 2007).

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 × yttrium concentration in the chyme)/ (nutrient concentration in the chyme × nutrient concentration in feed)]. Water flux (ml g^{-1} 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)].

2.2.6 Performance

Growth performance was calculated as described by (Saravanan et al., 2013b). The total feed intake per fish (FI, g $fish^{-1}$) was calculated as FI = (total

DM offered feed - uneaten feed)/ (fish number). Total fish weight gain (Wg, g $fish^{-1}$) was calculated as the difference between the average individual final (Wf) and initial (Wi) body weight per fish. The specific growth rate (SGR, % day^{-1}) was calculated as the ln(Wf) - ln(Wi))/ t*100. The feed conversion ratio (FCR) was calculated as FI/ Wg.

2.2.7 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 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 (GML) 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.

2.3 Results

2.3.1 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^{-1}), SGR (% day^{-1}) and FCR were unaffected by dEB ($p > 0.05$) and body weight increased from 306 g to 499 g during the experiment. As intended, total feed intake per fish (g fish^{-1}) was equal between treatments ($p > 0.05$).

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^{-1})	165	161	0.53	ns
Weight gain (g fish^{-1})	201	185	5.43	ns
SGR (% day^{-1})	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, $p > 0.05$.

2.3.2 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 focused on the main effect of diet in this results section (Figure 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 (Figure 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, middle, and distal intestine, respectively (Figure 1). In both stomach and proximal intestine, chyme DM was lower at high dEB diet than at the low dEB ($p < 0.01$; Figure

2.1A). Chyme DM in the mid and distal intestine were similar between treatments ($p > 0.05$).

Chyme pH was lowest in the stomach and increased as digesta passed through the intestine (Figure 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 ($p > 0.05$; Figure 1C) and was similar across GIT segments. Averaged over diets and time, osmolality was 392, 395, 374 and 380 mmol 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 (Figure 1D). Only in the stomach, water fluxes were influenced by both diet and time postprandial ($p < 0.01$), but no interaction was present (Figure 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 versus 2.3 ml per g^{-1} ingested DM). In the stomach, the water influx increased between 3 and 7 hours 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 versus 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 ($p > 0.05$; Figure 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^{-1} ingested DM in the middle and distal intestine, respectively.

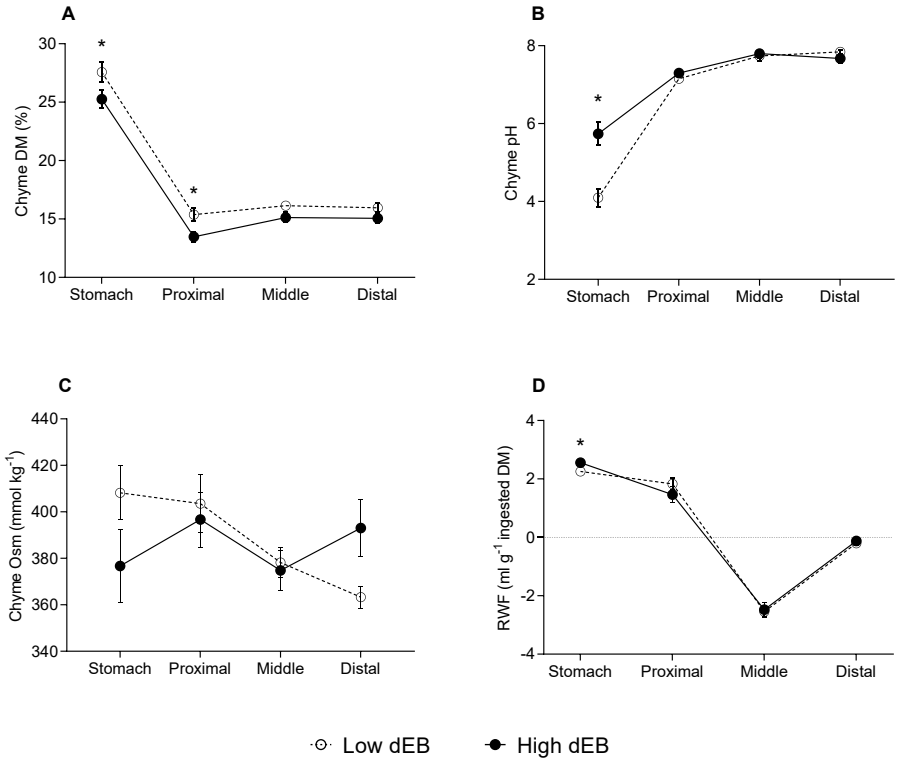

Figure 1. Chyme parameters and relative water fluxes as affected by dietary electrolyte balance (dEB) measured in the stomach, proximal, middle, and distal intestine. Values per diet are averaged over both sampling moments (n = 6). Figure legend: (A) chyme dry matter, DM; (B) chyme pH; (C) chyme osmolality, 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.

2.3.3 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 ($p < 0.05$; Figure 2A). Na^+ efflux occurred in the stomach, as indicated by the negative flux. In the stomach, Na^+ efflux was larger in fish fed the high dEB diet than those fed the low dEB diet (4.5 versus -1.1 mg g^{-1} ingested feed DM; Figure 2A). In the intestine, Na^+ fluxes were not different between diets ($p > 0.05$). Na^+ influx occurred in

the proximal intestine, whereas Na^+ efflux occurred in the middle and distal intestine (Figure 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 ($p > 0.05$; Figure 2B, Supplementary table S2). K^+ efflux took place in the stomach, proximal and middle intestine, while influx of K^+ 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.

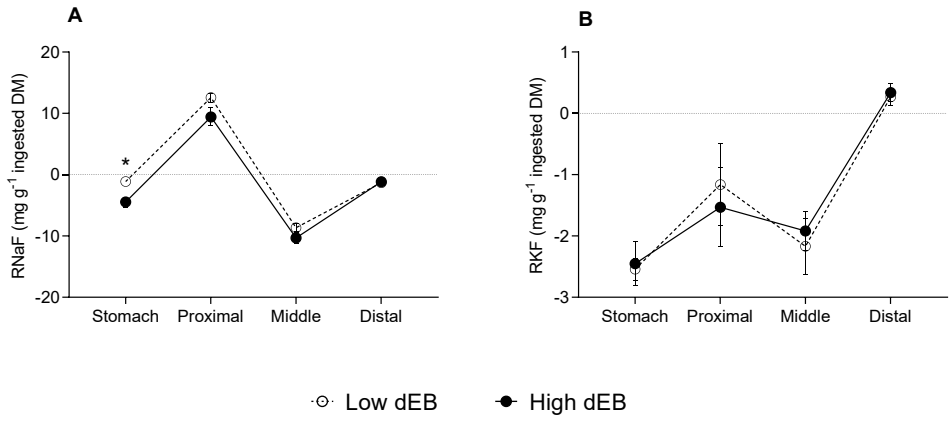

Figure 2. Mineral fluxes as affected by dietary electrolyte balance (dEB) measured in the stomach, proximal, middle, and distal intestine. Figure legend: (A) relative sodium fluxes (RNaF); (B) relative potassium fluxes (RKF); Values are means (n = 6) and standard error of the mean (SEM). Significant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

2.3.4 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 ($p > 0.05$).

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, $p > 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

2.3.5 Progression of digestion

Figure 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. Protein (CP) digestibility in all GIT segments were not different between 3 and 7 hours 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 towards the distal intestine (Figure 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 treatment in 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^{2+}) 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^{2+} digestibility was higher in all the GIT segments in fish fed the low dEB diet compared to the high dEB diet except for the proximal intestine (Figure 3B, Supplementary table S3). In the proximal intestine, Ca^{2+} ADC were similar between diets. With progression

of chyme from stomach to the proximal intestine Ca^{2+} ADC declined. Averaged over time, Ca^{2+} ADC was higher ($p < 0.05$) for low dEB fed fish compared to high dEB in the stomach (22.6 versus 14.3%). In the middle and distal intestine, Ca^{2+} 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^{2+} ADC between low and high dEB was more pronounced ($p < 0.01$) at 3 hours postprandial (28.6 versus 6.8%) then at 7 hours postprandial (15.2 versus 7.5%), whereas in the distal intestine it was more pronounced at 7 hours postprandial (31.5 versus 8.0%) than at 3 hours postprandial (10.7 versus 9.7%).

Phosphorus (P^{+}) digestibility was unaffected by diet, time and diet-time interaction in the stomach, proximal and distal intestine (Figure 3C, Supplementary table S3). In the middle intestine, P^{+} digestibility was 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 hours postprandial (51.4 versus 40.9%) becoming stable at 7 hours postprandial (37.0 versus 39.8%). The same trend was observed in the distal intestine, but this difference was not significant, which is 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 (Figure 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 versus -38%). In the middle and distal intestine, the difference in Na^{+} ADC between 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 (Figure 3D).

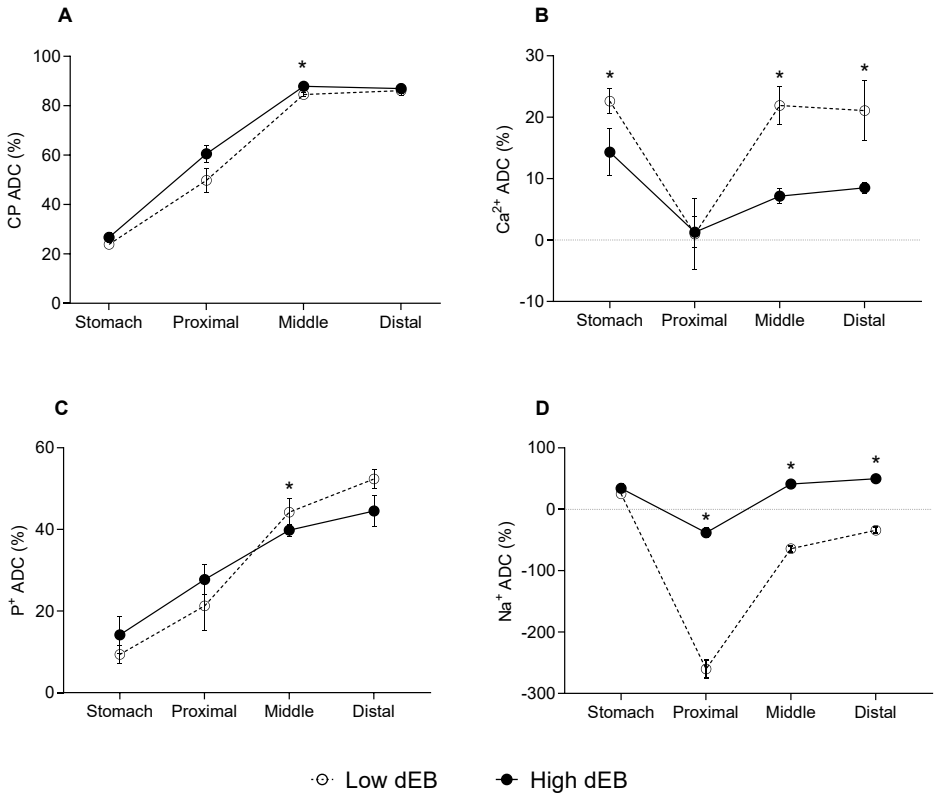

Figure 3. Progression of digestion (ADC) as affected by dietary electrolyte balance (dEB) measured in the stomach, proximal, middle, and distal intestine. Values per diet are averaged over both sampling moments (n = 6). Figure legend: (A) crude protein, CP; (B) calcium, Ca^{2+}; (C) phosphorus, P^{+}; (D) Sodium, Na^{+}; Significant difference between the two treatments 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 S3.

2.4 Discussion

This study shows 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) on 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 attributed to the different experimental design (fed versus 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 of 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., 2013b). Previous studies likewise showed higher chyme liquefaction in the stomach along with increasing dEB resulting in higher crude protein digestibility (Magnoni et al., 2018a; Saravanan et al., 2013b). 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. Similarly, in the present study, water influx in the stomach was higher in fish fed the high dEB diet, and CP digestibility was higher in all GIT segments. Despite a larger water influx in the stomach of fish fed a high dEB diet, chyme pH increased. As a result, we propose that water influx rather than chyme pH promote protein digestibility in the stomach (quicker movement of solubilized proteins from the stomach to the proximal intestine).

Diet composition is known to influence stomach pH (Magnoni et al., 2018a; Saravanan et al., 2013b). 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^+ uptake may lower the pH in the stomach. Furthermore, when Na^+ is taken up, the osmotic pressure in the gut lumen rises, stimulating water absorption (Bogé et al., 1988; Bucking and Wood, 2006b). Despite increased Na^+ uptake, 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., 2013b) and rainbow trout (Magnoni et al., 2018a), 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 reduced 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 (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^{-1} (Dabrowski et al., 1986; Grosell et al., 2000). Bucking and Wood (2006b) 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^+ efflux 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 show 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 part of the gut (middle and distal intestine). The Na^+ flux is linked to the K^+ flux by the Na^+-K^+-Cl^--cotransporter (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^{-1}) (Bucking and Wood, 2006b), dietary K^+ was almost completely absorbed from the stomach to the intestine, except for 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 contrasts 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.

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2.5 Supplementary tables

Supplementary table S1. Chyme dry matter (DM), pH, osmolality, and relative water fluxes (RWF) in the stomach, proximal, middle, and distal intestine of freshwater rainbow trout (*Oncorhynchus mykiss*) as affected by contrasting levels of dietary electrolyte balance (dEB) and time postprandial (3 and 7 hours).

		3 hours		7 hours			*p-values*		
		Low dEB	High dEB	Low dEB	High dEB	SEM	dEB	Time	dEB*Time
Chyme DM (%)	Stomach	29.4	26.2	25.8	24.3	0.84	**	**	ns
	Proximal	14.2	13	16.5	13.9	0.54	**	**	ns
	Middle	15.6	14.7	16.8	15.6	0.48	ns	ns	ns
	Distal	15.4	14.6	16.6	15.5	0.58	ns	ns	ns
Chyme pH	Stomach	4.4	6.1	3.6	5.4	0.40	**	ns	ns
	Proximal	7.3	7.3	7.2	7.3	0.11	ns	ns	ns
	Middle	7.8	7.8	7.9	7.8	0.15	ns	ns	ns
	Distal	7.9	7.6	7.9	7.7	0.16	ns	ns	ns
Chyme osmolality (mmol kg^{-1})	Stomach	424	397	390	356	19.7	ns	ns	ns
	Proximal	391	403	394	390	15.7	ns	ns	ns
	Middle	370	378	375	372	10.9	ns	ns	ns
	Distal	369	394	364	392	15.6	ns	ns	ns
RWF (ml g^{-1} ingested DM)	Stomach	2.1	2.5	2.4	2.7	0.09	**	**	ns
	Proximal	1.7	1.8	2	1.1	0.34	ns	ns	ns
	Middle	-2.2	-2.7	-2.9	-2.3	0.32	ns	ns	ns
	Distal	-0.1	-0.1	-0.3	-0.2	0.12	ns	ns	ns

SEM, standard error of mean; ns, not significant, $p > 0.05$; **, $p < 0.01$; mmol kg^{-1}, millimol per kilogram; ml water g^{-1} ingested DM, milliliters of water per gram of ingested dry matter.

Supplementary table S2. Relative ion fluxes in the stomach, proximal, middle, and distal intestine of freshwater rainbow trout (*Oncorhynchus mykiss*) as affected by contrasting levels of dietary electrolyte balance (dEB) and time postprandial (3 and 7 hours).

		3 hours		7 hours			*p-values*		
		Low dEB	High dEB	Low dEB	High dEB	SEM	dEB	Time	dEB*Time
Relative Na^+ Fluxes (mg g^{-1} ingested DM)	Stomach	-0.9	-3.7	-1.3	-5.2	0.83	**	ns	ns
	Proximal	12.1	10.1	13.1	8.7	1.78	ns	ns	ns
	Middle	-8.6	-11.6	-8.7	-9	1.10	ns	ns	ns
	Distal	-0.9	-1.1	-1.7	-1.2	0.38	ns	ns	ns
Relative K^+ Fluxes (mg g^{-1} ingested DM)	Stomach	-2.2	-2.1	-2.9	-2.8	0.37	ns	ns	ns
	Proximal	-2.2	-1.6	-0.1	-1.5	0.89	ns	ns	ns
	Middle	-1.6	-2.2	-2.8	-1.6	0.53	ns	ns	ns
	Distal	0.2	0.5	0.3	0.2	0.21	ns	ns	ns

dEB, dietary electrolyte balance; SEM, standard error of mean; ns, not significant, $p > 0.05$; **, $p < 0.01$; mg Na g^{-1} ingested DM, milligrams of sodium per gram of ingested dry matter; mg K^+ g^{-1} ingested DM, milligrams of potassium per gram of ingested dry matter.

Supplementary table S3. Progression of digestion of crude protein (CP ADC), calcium (Ca^{2+} ADC), potassium (P^{+} ADC), and sodium (Na^{+}) in the stomach, proximal, middle, and distal intestine of freshwater rainbow trout (*Oncorhynchus mykiss*) as affected by contrasting levels of dietary electrolyte balance (dEB) and time postprandial (3 and 7 hours).

		3 hours		7 hours			*p-values*		
		Low dEB	**High dEB**	**Low dEB**	**High dEB**	**SEM**	**dEB**	**Time**	**dEB*Time**
CP ADC (%)	Stomach	23.3	25.8	24.5	27.6	2.28	ns	ns	ns
	Proximal	57.8	58.3	41.7	62.7	4.98	ns	ns	ns
	Middle	85.8	88	83.3	87.8	0.93	**	ns	ns
	Distal	87.8	85.8	84.5	88.1	2.82	ns	ns	ns
Ca^{2+} ADC (%)	Stomach	18.8	8.8	26.4	19.9	3.53	*	*	ns
	Proximal	10.4	1.2	-8.5	1.4	5.19	ns	ns	ns
	Middle	28.6^{c}	6.8^{a}	15.2^{b}	7.5^{ab}	1.80	***	**	**
	Distal	10.7^{a}	9.1^{a}	31.5^{b}	8^{a}	2.00	***	**	***
P^{+} ADC (%)	Stomach	5.5	9.7	13.2	18.6	4.72	ns	ns	ns
	Proximal	30.2	24.7	12.3	30.8	6.33	ns	ns	ns
	Middle	51.4^{b}	40.9^{a}	37^{a}	39.8^{a}	1.66	*	**	**
	Distal	56.4	43.7	48.4	45.4	4.59	ns	ns	ns
Na^{+} ADC (%)	Stomach	20.0	28.6	30.5	39.9	6.84	ns	ns	ns
	Proximal	-255	-49.3	-266	-27.1	18.1	***	ns	ns
	Middle	-60.3	40.1	-67.9	42.2	5.59	***	ns	ns
	Distal	-38.9	48.1	-29.6	51.8	5.50	***	ns	ns

SEM, standard error of mean; ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Means within the same row not sharing a common superscript are significantly different ($p < 0$)

CHAPTER 3

Effect of dietary macronutrient composition and buffering capacity on chyme characteristics and digestion kinetic 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 hours). 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.

3.1 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., 2001). Feed BC is affected by its composition (protein, fat, minerals, and organic acids) and 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. Plant ingredients have a lower BC than fish meal (Giger-Reverdin et al., 2001; 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) 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, Krogdhal 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 (NRC, 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 $CaCO_3$.

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.

3.2.1. 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 the chyme sampling moment at the end of the experiment. Chyme was sampled at 3 and 7 hours 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 grams was collected from both diets and stored at 4 °C for analysis.

Table 1. Ingredients and analyzed nutrient composition of 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
$CaCl_2{*}2H_2O$	0.5	0.5
Yttrium oxide	0.02	0.02

P:E, protein to energy ratio; $CaCO_3$, buffering capacity.

Table 1 continued. Ingredients and analyzed nutrient composition of experimental diets.

	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^{-1} 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[2]	366	363	205	196
Crude ash	53.5	76.6	64.9	86.7
Phosphorus	10.7	10.4	11.6	11.6
Calcium	7.8	19.4	7.7	19.5
Sodium	2.9	2.7	4.6	4.2
Potassium	4.8	4.5	6.5	6.4
GE (mg kg^{-1} DM)	22.2	21.4	21.8	21.1
CP:GE (mg kJ^{-1})	16.1	16.2	26.4	26.4
DP:DE (mg kJ^{-1})	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)[3]	0.212	0.328	0.284	0.406
Alkaline BC (mmol NaOH/g feed)[4]	0.174	0.218	0.230	0.260

P:E, protein to energy ratio; $CaCO_3$, buffering capacity; 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^{-1} 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 $CaIO_3 6H_2O$), 2 mg.

[2]Starch analyses included the free sugar fraction.

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

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

3.2.2. 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 24 tanks with 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 measured daily 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^{-1}; 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^{-1}, 0.2 mg l^{-1}, and 500 mg l^{-1}, respectively.

3.2.3. 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 hour maximum and the 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). 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 minutes 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 was adjusted based on the remaining number of fish in the respective tank(s).

3.2.4. 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^{-1}) 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.

3.2.5. 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^{2+}, 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 ºC) 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^{2+}, 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).

3.2.6. Calculations

Fish performance was measured over the 42- to 44-day period. 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, % day^{-1})

was calculated as, (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^{2+}, 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)].

3.2.7. 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 hours) 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.

3.3 Results

3.3.1 Fish performance

Fish performance is depicted in Table 2. All diets were readily consumed and feed intake (g DM $fish^{-1}$) was similar between treatments ($p > 0.05$). Fish mean body weight increased from 284 g to 564 g during the experiment. Weight gain (g $fish^{-1}$) and SGR (% day^{-1}) 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%.

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 $fish^{-1}$)	244	246	248	244	0.03	ns	ns	ns
Weight gain (g $fish^{-1}$)	271	259	302	284	4.02	***	**	ns
SGR (% day^{-1})	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 ($n = 6$).

3.3.2 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 Figure 1. The full 3-way ANOVA results with all treatments means including sampling time are reported in the Supplementary table S1. DM contents of all diets were similar, ranging between 92-94% (Table 1).

Chyme DM (Figure 1A, 1D) 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 (Figure 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 (Figure 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^{-1} 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^{-1} 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 (Figure 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) (Figure 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) (Figure 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) (Figure 1E).Due to the 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 (Figure 1F). In contrast, 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 (Figure 1C).

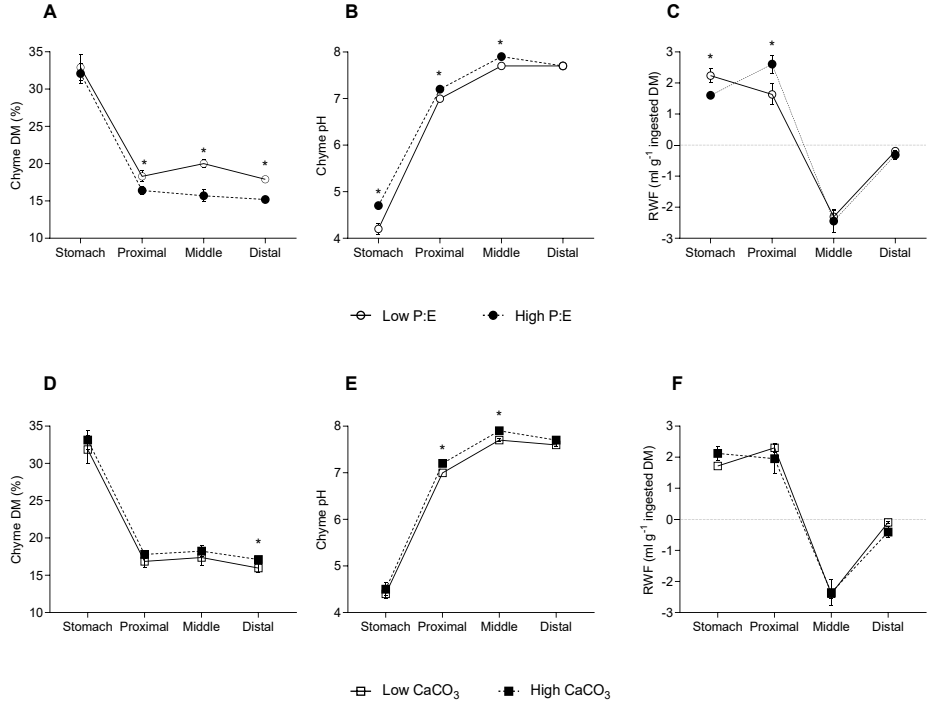

Figure 1. Chyme dry matter (DM), pH and relative water fluxes as affected by the main effects of dietary protein to energy ratio (P:E) and calcium carbonate levels ($CaCO_3$) in the stomach, proximal, middle, and distal intestine. (A) chyme DM, (B) chyme pH and (C) RWF as affected by dietary P:E ratio. (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 standard error of mean (SEM). Mean values and level of significance are given in Supplementary table S1.

3.3.3. Electrolyte fluxes

Time and interactions effects with time were minimal for electrolyte fluxes (Ca^{2+}, 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 Figure 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 (Figure 2). Relative Ca^{2+} flux (RCaF) was negative in the stomach (efflux), but it was not affected by the dietary P:E ratio (Figure 2A). RCaF was positive in the proximal intestine (influx), and it increased ($p < 0.05$) in fish fed the low P:E diet. Net Ca^{2+} efflux occurred in the middle intestine, while a minor Ca^{2+} influx occurred in the distal intestine, but no dietary effect was detected. Relative Na^{+} flux (RNaF) was negative (efflux) in all GIT segments except for the proximal intestine (Figure 2B, 2E). RNaF was only affected by dietary P:E ratio in the distal intestine (Figure 2B). In the distal intestine, RNaF was lower ($p < 0.05$) in fish fed the high P:E diet compared to low P:E diet. Relative K^{+} flux (RKF) was negative (efflux) in all the segments of the GIT, except for the distal intestine (Figure 2C, 2F). 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 (Figure 2C).

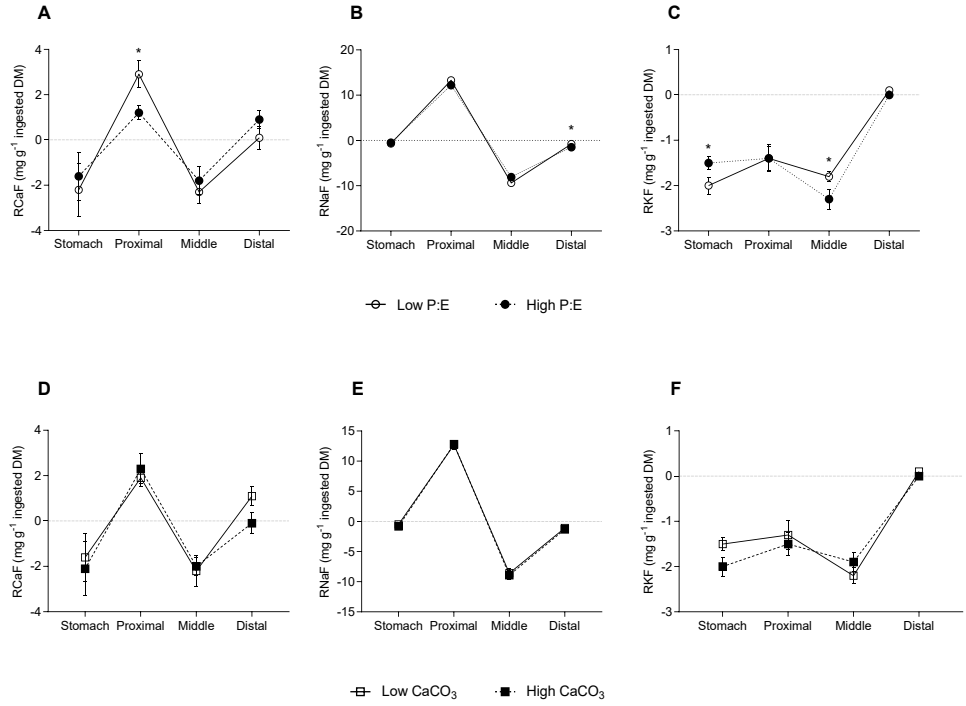

Figure 2. Relative calcium fluxes (RCaF), relative sodium fluxes (RNaF) and relative potassium fluxes (RKF) as affected by the main effects of dietary protein to energy ratio (P:E) and calcium carbonate levels ($CaCO_3$) in the stomach, proximal, middle, and distal intestine. (A) RCaF, (B) RNaF and (C) RKF as affected by dietary P:E ratio. (D) RCaF, (E) RNaF and (F) RKF 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 standard error of mean (SEM). Mean values and level of significance are given in Supplementary table S2.

3.3.4. Apparent digestibility

Nutrient apparent digestibility coefficients (ADCs) were affected both dietary factors (Table 3). Except for phosphorus digestibility (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^{2+} 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^{2+} 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^{2+}, P^{+} and K^{+} digestibility compared to the low $CaCO_3$ diet.

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)

3.3.5. Progression of digestion

Figure 3 depicts the progressive digestibility values of crude protein (CP ADC) and phosphorus (P^{+} ADC) as affected by the contrasting dietary P:E 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 (Figure 3). Numerically, CP ADC kinetic was higher in fish fed a low P:E diet in all GIT segments except the distal intestine (Figure 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 (Figure 3B, 3D).

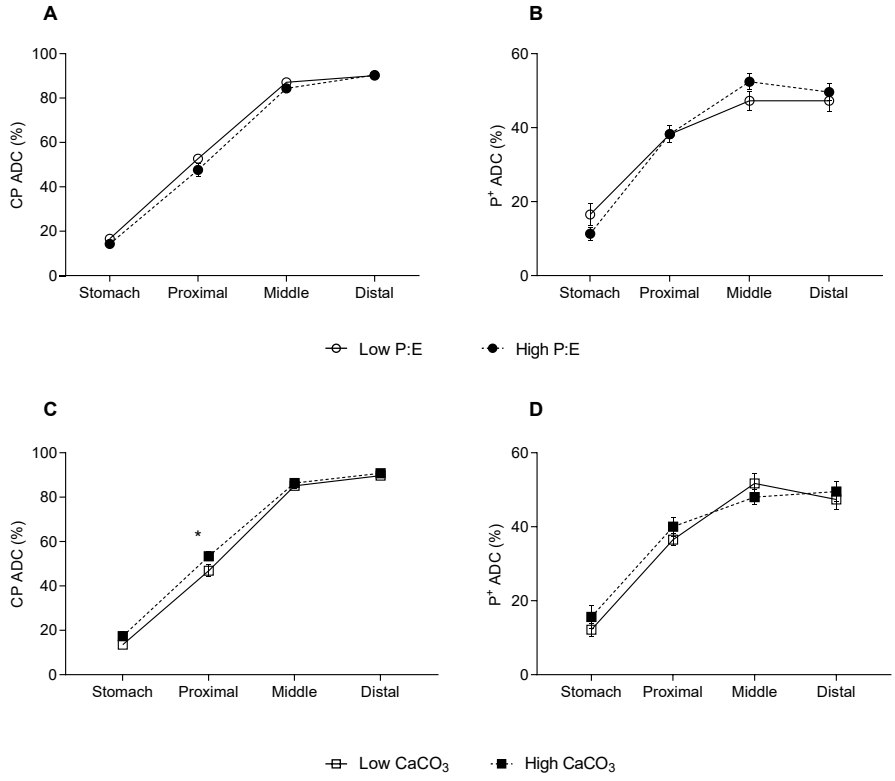

Figure 3. Progression of digestion of crude protein (CP ADC) and phosphorous (P^+ ADC) as affected by the main effects of dietary protein to energy ratio (P:E) and calcium carbonate levels ($CaCO_3$) in the stomach, proximal, middle, and distal intestine. (A) CP ADC, (B) P^+ ADC as affected by dietary 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 standard error of mean (SEM). Mean values and level of significance are given in Supplementary table S3.

3.4 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., 2001; 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 fish. 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 it affected dietary BC, although it was much smaller than 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 (Figure 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., 2013b). 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 (Figure 3A). One 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 delays 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 contrasting protein levels in the diet can also 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 significant 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 CP digestion kinetic was slightly lower in fish fed the high P:E diet, this was reversed in the faecal digestibility (ADC). Earlier 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^{2+} digestibility. The protein mixture used in the current study in the diet formulation was fully plant-based ingredients. Therefore, the lower faecal Ca^{2+} 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., 2001a).

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 (Figure 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^{2+} 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, 2006b; 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 influenced 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 influences 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., 2022b; Parma et al., 2019). 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.

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3.5 Supplementary tables

Supplementary table S1. Chyme dry matter (DM), pH, osmolality, and relative water fluxes (RWF) measured in the stomach, proximal, middle, and distal intestine of freshwater rainbow trout (*Oncorhynchus mykiss*) as affected by contrasting levels of dietary protein to energy ratio (P:E), calcium carbonate ($CaCO_3$) and time postprandial (3 and 7 hours).

		3 hours				7 hours											
		Low P:E		High P:E		Low P:E		High P:E									
											p-values						
		Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	pSEM	P:E	$CaCO_3$	Time	P:E* $CaCO_3$	P:E* Time	$CaCO_3$* Time	P:E* $CaCO_3$* Time
Chyme DM (%)	Stomach	37.7	36.7	35.5	37.2	26.9	30.3	27.4	28.3	2.09	ns	ns	***	ns	ns	ns	ns
	Proximal	20.0	18.8	16.0	16.1	16.0	18.4	15.4	18.0	1.24	*	ns	ns	ns	ns	ns	ns
	Middle	18.6	20.3	16.5	16.7	20.9	19.9	13.5	16.0	1.41	***	ns	ns	ns	ns	ns	ns
	Distal	16.7	18.1	14.5	15.5	18.4	18.3	14.3	16.6	0.72	***	*	ns	ns	ns	ns	ns
Chyme pH	Stomach	4.4	4.5	4.7	4.9	4.1	3.9	4.4	4.6	0.16	***	ns	**	ns	ns	ns	ns
	Proximal	6.8	7.0	7.2	7.4	6.9	7.2	7.1	7.3	0.09	***	*	ns	ns	ns	ns	ns
	Middle	7.6	7.6	7.8	8.0	7.6	7.8	7.8	8.0	0.07	***	*	ns	ns	ns	ns	ns
	Distal	7.6	7.7	7.7	7.8	7.6	7.8	7.7	7.6	0.09	ns	ns	ns	ns	ns	ns	ns
Chyme osmolality (mmol kg^{-1})	Proximal	453	448	458	444	417	418	430	415	16.9	ns	ns	*	ns	ns	ns	ns
	Middle	397	401	403	403	366	387	385	382	10.1	ns	ns	**	ns	ns	ns	ns
	Distal	391	378	388	391	374	374	380	398	13.4	ns	ns	ns	ns	ns	ns	ns
RWF (ml g^{-1} ingested DM)	Stomach	1.7	2.2	1.5	1.6	2.1	2.9	1.5	1.8	0.31	*	ns	ns	ns	ns	ns	ns
	Proximal	2.6	1.4	2.2	2.5	1.9	0.6	2.4	3.3	0.60	*	ns	ns	*	ns	ns	ns
	Middle	-2.6	-1.8	-2.1	-2.5	-2.7	-2.1	-2.2	-3.0	0.67	ns	ns	ns	ns	ns	ns	ns
	Distal	-0.1	-0.5	-0.1	-0.1	-0.1	-0.1	-0.1	-0.9	0.25	ns	ns	ns	ns	ns	ns	ns

All the analyses were done by a 3-way ANOVA. Values are means (n = 3) and pooled standard error of the mean (pSEM). ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. mmol kg^{-1}, milliosmoles/kilogram; ml water g^{-1} ingested DM, milliliters of water per gram of ingested dry matter.

Supplementary table S2. Relative ion fluxes in the stomach, proximal, middle, and distal intestine of freshwater rainbow trout (*Oncorhynchus mykiss*) as affected by contrasting levels of dietary protein to energy ratio (P:E), calcium carbonate ($CaCO_3$) and time postprandial (3 and 7 hours).

		3 hours				7 hours											
		Low P:E		High P:E		Low P:E		High P:E			*p-values*						
		Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	pSEM	P:E	$CaCO_3$	Time	P:E* $CaCO_3$	P:E* Time	$CaCO_3$* Time	P:E*$CaCO_3$* Time
RCaF (mg g^{-1} ingested DM)	Stomach	-0.4	-2.4	-5.0	-0.2	-0.4	-5.5	-0.7	-0.4	2.2	ns	ns	ns	ns	ns	ns	ns
	Proximal	2.1	4.6	2.1	0.9	2.7	2.3	0.6	1.4	1.0	*	ns	ns	ns	ns	ns	ns
	Middle	-3.4	-2.3	-0.1	-2.9	-2.7	-0.8	-2.4	-1.8	1.1	ns	ns	ns	ns	ns	ns	ns
	Distal	1.3	-1.3	1.3	0.5	-0.1	0.6	1.7	-0.1	0.9	ns	ns	ns	ns	ns	ns	ns
RNaF (mg g^{-1} ingested DM)	Stomach	-0.4	-1.0	-0.6	-0.4	-0.6	-0.9	-0.3	-0.7	0.22	ns	ns	ns	ns	ns	ns	ns
	Proximal	13.7	14.6	10.5	12.6	14.3	10.8	12.3	13.3	1.24	ns	ns	ns	ns	ns	ns	ns
	Middle	-9.3	-10.6	-6.4	-8.9	-10.5	-7.3	-8.3	-8.8	1.40	ns	ns	ns	ns	ns	ns	ns
	Distal	-0.9	-1.0	-1.5	-1.3	-0.7	-0.7	-1.3	-2.0	0.26	**	ns	ns	ns	ns	ns	ns
RKF (mg g^{-1} ingested DM)	Stomach	-1.4	-2.4	-1.5	-1.2	-1.9	-2.4	-1.3	-1.9	0.326	*	ns	ns	ns	ns	ns	ns
	Proximal	-1.3	-1.4	-0.3	-1.2	-1.7	-1.1	-1.9	-2.1	0.562	ns	ns	ns	ns	ns	ns	ns
	Middle	-2.1	-1.6	-3.0	-2.9	-1.7	-1.5	-1.8	-1.5	0.226	**	ns	***	ns	**	ns	ns
	Distal	0.15	0.13	0.06	0.04	0.12	0.06	0.04	-0.04	0.069	ns	ns	ns	ns	ns	ns	ns

RCaF, relative calcium fluxes; RNaF, relative sodium fluxes; RKF, relative potassium fluxes. All the analyses were done by a 3-way ANOVA. Values are means (n = 3) and pooled standard error of the mean (pSEM). ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. mg g^{-1} ingested DM, milligrams per gram of ingested dry matter.

Supplementary table S3. Progression of digestion of crude protein (CP ADC) and potassium (P^+ ADC) in the stomach, proximal, middle, and distal intestine of freshwater rainbow trout (*Oncorhynchus mykiss*) as affected by contrasting levels of dietary protein to energy ratio.

		3 hours				7 hours											
		Low P:E		High P:E		Low P:E		High P:E						*p-values*			
%		Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	Low $CaCO_3$	High $CaCO_3$	pSEM	P:E	$CaCO_3$	Time	P:E* $CaCO_3$	P:E* Time	$CaCO_3$* Time	P:E*$CaCO_3$* Time
CP ADC	Stomach	12	21	15	15	14	20	13	14	3.90	ns	ns	ns	ns	ns	ns	ns
	Proximal	44	54	40	45	55	58	49	57	3.95	ns	*	**	ns	ns	ns	ns
	Middle	84	88	85	83	88	89	84	86	2.23	ns	ns	ns	ns	ns	ns	ns
	Distal	89	90	89	89	90	92	91	92	1.28	ns	ns	ns	ns	ns	ns	ns
P^+ ADC	Stomach	11.0	19.3	16.3	5.9	11.5	24.1	9.9	13.0	4.62	ns	ns	ns	*	ns	ns	ns
	Proximal	37.3	37.1	34.3	31.0	31.4	46.8	42.8	45.0	3.38	ns	ns	*	ns	ns	*	ns
	Middle	54.4	42.5	56.4	48.6	40.0	52.5	56.0	48.6	4.21	ns	ns	ns	ns	ns	ns	ns
	Distal	49.2	44.8	52.2	45.5	38.9	56.4	49.4	51.5	5.24	ns	ns	ns	ns	ns	ns	ns

(P:E), calcium carbonate ($CaCO_3$) and time postprandial (3 and 7 hours).

All the analyses were done by a 3-way ANOVA. Values are means (n = 3) and pooled standard error of the mean (pSEM). ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$.

CHAPTER 4

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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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 were 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.

4.1 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, 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 of 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 hours of feeding. Nevertheless, whether the water fluxes were 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.

4.2 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.

4.2.1 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}), at salinity according to the assigned treatment. The photoperiod set at 12:12, L:D. Water temperature was kept at 12 ºC and oxygen saturation in the outlet was kept above 80%. At the start of the experiment, the 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 hours each feeding) for 8 weeks using automatic feeders until apparent satiation and feed intake was monitored through collection of feed spills. The uneaten feed pellets were collected 15 minutes 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 over time.

Table 1. Ingredients and analyzed 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

[1]Vitamin premix (giving the following concentrations per kg diet): vitamin A: 3000 IU; vitamin D3: 3800 IU; vitamin E: 300 mg; vitamin K3: 30 mg; vitamin B1: 30 mg; vitamin B2: 45 mg; vitamin B6: 38 mg; vitamin B12: 0.08 mg; niacin: 300 mg; Ca-D-pantonat: 90 mg; biotin: 1.5 mg; folic acid: 15 mg; vitamin C: 300 mg. Mineral premix (giving the following concentrations per kg diet): Mg, 0.1 g; Fe: 100 mg, Mn: 30 mg, Zn:130 mg, Cu: 6 mg, I: 5 mg, Co: 0.05 mg, Se: 0.3 mg.

4.2.2 Sampling

Fish were sampled 6 hours 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 for 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 x 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 minutes 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.

4.2.3 Analyses and calculations

The diets were homogenised and analyzed for dry matter, ash, lipid, and protein following standard procedures. Briefly, dry matter was measured after drying at 105 ºC for 24 h; ash content determined by combustion in a muffle furnace at 550 ºC 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., 2013b). 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).

4.2.4 Statistical analyses

Fish tanks (n = 16) were used as experimental units for all analysed parameters and data are expressed as the mean ± standard errors of the mean (SEM) or standard deviations (SD) per treatment of four replicates. All parameters were tested for the effect of salinity by regression analysis and one-way ANOVA. Treatment means and results of one-way ANOVA are given in the supplementary tables (Section 5). 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 the 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.

4.3 Results

During the adaption period, feed intake was negatively affected by increasing water salinity ($p < 0.05$) (Supplementary able 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%.

4.3.1 Chyme characteristics and relative water fluxes

Chyme characteristics and relative water fluxes (RWF) as affected by water salinity are depicted in Figure 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 ($p < 0.01$) (Figure 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 (Figure 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 (Figure 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 (Figure 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.

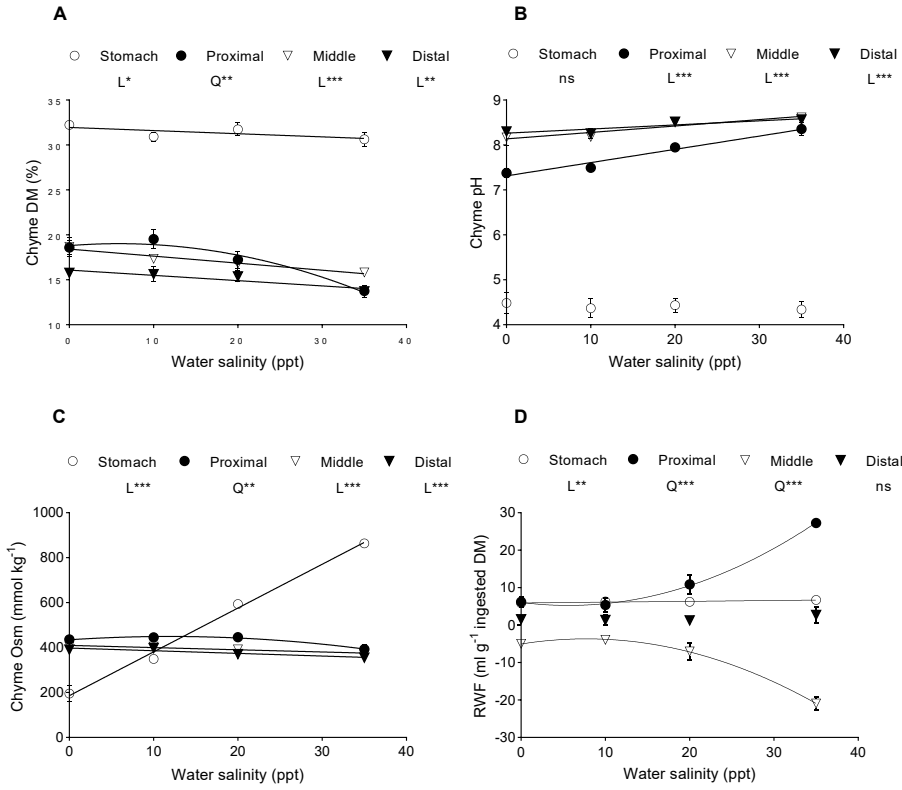

Figure 1. (A) Chyme dry matter, DM, (B) pH, (C) osmolality, Osm and (D) relative water fluxes, RWF, as affected by increasing water salinity (0, 10, 20 and 35 ppt) in the stomach, proximal, middle, and distal intestine of Atlantic salmon. Solid lines indicate a significant relationship, either linear (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 standard deviation (SD). Estimations of the significant linear or quadratic relationships are given in Supplementary table S3.

4.3.2 Electrolyte fluxes

Ion fluxes in the GIT as affected by water salinity are depicted in Figure 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 (Figure 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 (Figure 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 (Figure 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 (Figure 2D; Supplementary table S4). The increase and decline of RKF in the stomach and proximal intestine were similar in absolute terms, as indicated by the estimated slopes being 0.01 and -0.01, respectively (Supplementary table S5).

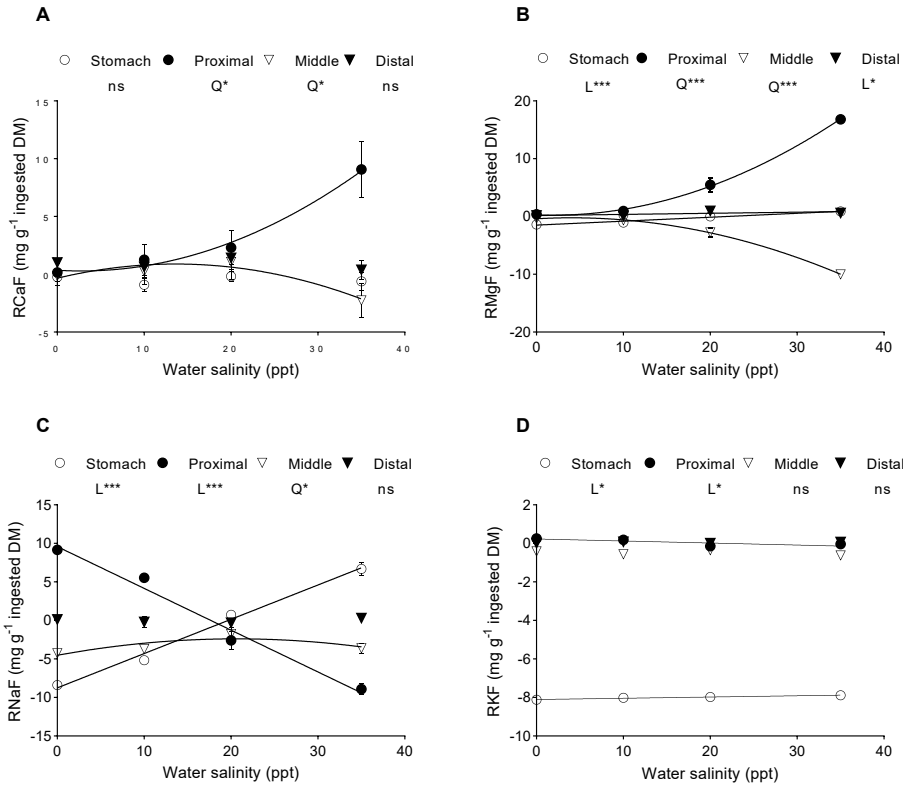

Figure 2. Relative fluxes of (A) calcium, (RCaF); (B) magnesium, (RMgF); (C) sodium, (RNaF) and (D) potassium, (RKF) in the stomach, proximal, middle, and distal intestine of Atlantic salmon, as affected by increasing water salinity (0, 10, 20 and 35 ppt). Solid lines indicate a significant relationship, 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 standard deviation (SD). Estimations of the significant linear or quadratic relationships are given in Supplementary table S6.

4.3.3 *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 20 ppt (142 mmol l^{-1}) and 35 ppt (139 mmol l^{-1}) (Table 2).

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 ($R^2 = 0.87$).

4.3.4 Crude protein digestion kinetic

Crude protein (CP) progression of digestion is depicted in Figure 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 (Figure 3).

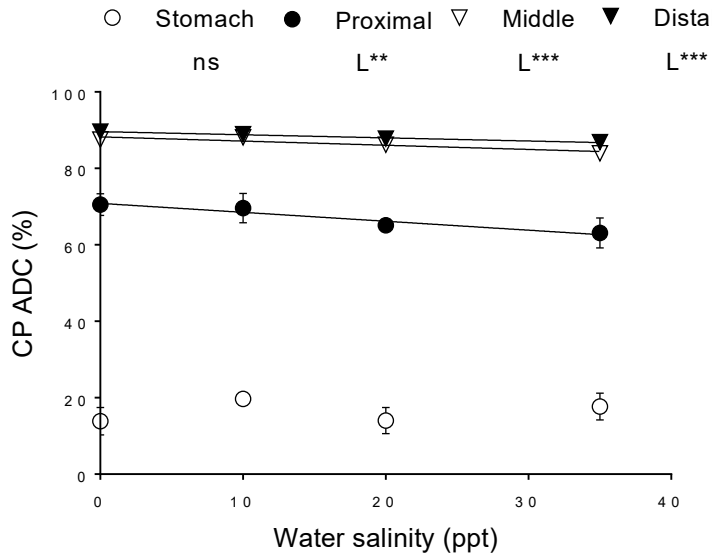

Figure 3. Progression of digestion of crude protein (CP ADC) as affected by increasing water salinity (0, 10, 20 and 35 ppt) in the stomach, proximal, middle, and distal intestine of Atlantic salmon smolt. Solid lines indicate a significant relationship, either linear (L) or quadratic (Q) (**, $p < 0.01$, ***, $p < 0.001$), while no lines indicate a non-significant relationship (ns, $p > 0.05$). Values are expressed as the mean per treatment (n = 4) standard deviation (SD). 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).

4.3.5 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**	**Regression**
							p-value	*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	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 ($R^2 = 0.50$).

4.4 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 contents 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 0.13 ml kg^{-1} h^{-1}, whereas fed fish drink nearly five times as much (about 0.6 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., 1998; 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 (Figure 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 et al., 2005; 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., 2022b). 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 (Figure 1B). Even though base secretions play a key 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 (Figure 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 (Figure 2A, 2B) 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 oesophagus. In fact, the efflux of monovalent ions increased with water salinity in the proximal intestine (Figure 2C, 2D). 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 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 (Figure 1A; Figure 3). Usher et al. (1990) and Krogdahl et al. (2015), however, found no significant differences in 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., 2012, 2013; 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 (Figure 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%) (Figure 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 affected chyme characteristics and digestion kinetics in the intestinal segments rather than the stomach, where the ingested food seems to stay longer (higher chyme DM). Therefore, we suggest that, at higher salinities, most of the ingested water is bypassing the stomach and moving to

the proximal intestine more rapidly compared to the chyme in the stomach (Figure 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.

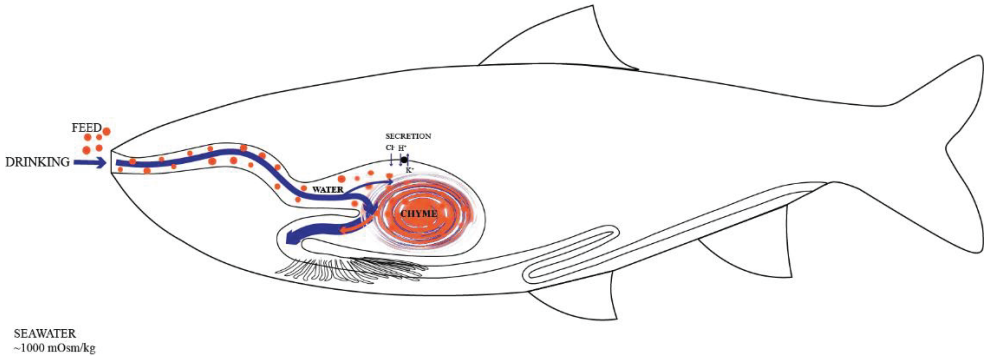

Figure 4. Water and chyme dynamic in the stomach of seawater acclimatized Atlantic salmon (*Salmo salar*).

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4.5 Supplementary tables

Supplementary table S1. Growth performance of Atlantic salmon smolt fed a commercial diet for 8 weeks 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*
Feed intake (g fish^{-1})	181^{b}	171^{ab}	127^{a}	140^{ab}	11.9	*	L*
Weight gain (g fish^{-1})	200^{b}	161^{b}	105^{a}	110^{a}	10.3	***	Q*
SGR (% day^{-1})	1.62^{c}	1.37^{b}	0.95^{a}	0.99^{a}	0.06	***	Q**
FCR	0.91^{a}	1.06^{b}	1.21^{c}	1.28^{d}	0.02	***	Q***
Survival (%)	100	99	98	99	-	-	-

SGR, specific growth rate; FCR, feed conversion ratio on dry matter basis. L, linear correlation; Q, quadratic correlation; ns, not significant, $p > 0.05$; *, $p < 0.05$; ***, $p < 0.001$. Values are expressed as the mean per treatment (n = 4) and pooled standard errors of the mean (pSEM).

Supplementary table S2. Chyme dry matter (DM), pH, osmolality, and relative water fluxes (RWF) in the stomach, proximal, middle, and distal intestine 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*
Chyme DM (%)	Stomach	32.3^{b}	30.9^{ab}	31.7^{ab}	30.6^{a}	0.33	*	L*
	Proximal	18.6^{bc}	19.5^{c}	17.2^{b}	13.8^{a}	0.45	***	Q**
	Middle	18.7^{b}	17.3^{ab}	16.8^{a}	15.8^{a}	0.37	**	L***
	Distal	15.8^{a}	15.7^{a}	15.4^{a}	13.7^{b}	0.34	**	L**
Chyme pH	Stomach	4.49	4.37	4.44	4.34	0.10	ns	ns
	Proximal	7.38^{a}	7.49^{a}	7.95^{b}	8.36^{c}	0.05	***	L***
	Middle	8.17^{a}	8.18^{a}	8.52^{b}	8.61^{b}	0.06	***	L***
	Distal	8.30^{a}	8.26^{a}	8.52^{b}	8.57^{b}	0.04	***	L***
Chyme osmolality (mmol kg^{-1})	Stomach	195^{a}	349^{b}	594^{c}	864^{d}	11.5	***	L***
	Proximal	436^{a}	448^{a}	446^{a}	403^{b}	7.31	**	Q**
	Middle	406^{b}	405^{b}	392^{ab}	380^{a}	4.87	*	L***
	Distal	391^{b}	390^{b}	371^{a}	359^{a}	4.41	**	L***
RWF (ml g^{-1} ingested DM)	Stomach	5.9^{a}	6.3^{ab}	6.2^{ab}	6.7^{b}	0.18	*	L**
	Prox	6.2^{a}	5.4^{a}	10.9^{b}	27.3^{c}	0.93	***	Q***
	Mid	-5.0^{b}	-3.9^{b}	-7.1^{b}	-20.9^{a}	0.80	***	Q***
	Distal	1.6	1.4	1.3	2.7	0.65	ns	ns

L, linear effect; Q, quadratic effect; ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values are expressed as the mean per treatment (n = 4) and the pooled standard errors of the mean (pSEM). Estimations of the significant linear or quadratic relationships are Supplementary table S3.

Supplementary table S3. The estimated linear (L) and quadratic (Q) relationships with water salinity (X) of the dependent variables (relative water flux, chyme dry matter, osmolality, and pH) depicted in Figure 1.

Dependent variables	Equation (intercept (SE) ± β (SE) ± β_1 (SE))	R^2	Regression *p-value*
Chyme dry matter (%)			
Stomach	Y = 32 (0.3) – 0.04 (0.2) X	0.27	L*
Proximal	Y = 19 (0.5) + 0.08 (0.07) X – 0.01 (0.002) X^2	0.85	Q**
Middle	Y = 18 (0.3) – 0.08 (0.02) X	0. 69	L***
Distal	Y = 16 (0.3) – 0.06 (0.02) X	0.54	L**
Chyme pH			
Stomach	4.4 (0.05)	-	ns
Proximal	Y = 7 (0.1) + 0.03 (0.002) X	0.92	L***
Middle	Y = 8 (0.1) + 0.01 (0.003) X	0.66	L***
Distal	Y = 8 (0.04) + 0.01 (0.002) X	0.60	L***
Chyme osmolality (mmol kg^{-1})			
Stomach	Y = 186 (12) + 19.5 (0.6) X	0.99	L***
Proximal	Y = 436 (6) + 2 (0.9) X – 0.1 (0.03) X^2	0.69	Q**
Middle	Y = 408 (4) – 0.8 (0.2) X	0.60	L***
Distal	Y = 393 (4) – 1 (0.2) X	0.72	L***
Relative water fluxes (ml g^{-1} ingested DM)			
Stomach	Y = 6 (0.1) + 0.02 (0.01) X	0.48	L**
Proximal	Y = 6.3 (0.68) – 0.4 (0.11) X + 0.03 (0.003) X^2	0.98	Q***
Middle	Y = -5 (0.7) + 0.4 (0.1) X – 0.03 (0.003) X^2	0.97	Q***
Distal	1.7 (0.32)	-	ns

DM, dry matter; L, linear effect; Q, quadratic effect. ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $p < 0.05$.

Supplementary table S4. Relative calcium, magnesium, sodium and potassium fluxes in the stomach, proximal, middle, and distal intestine of Atlantic salmon smolt, as affected by increasing water salinity (0, 10, 20 and 35 ppt).

mg g^{-1} ingested DM		0 ppt	10 ppt	20 ppt	35 ppt	pSEM	ANOVA *p-value*	Regression *p-value*
Relative calcium flux	Stomach	-0.2	-0.9	-0.2	-0.6	0.32	ns	ns
	Proximal	0.2^{a}	1.3^{a}	2.3^{a}	9.1^{b}	0.80	***	Q*
	Middle	-0.1^{ab}	0.3^{ab}	1.1^{b}	-2.3^{a}	0.62	*	Q*
	Distal	1.0	0.6	1.4	0.4	0.44	ns	ns
Relative magnesium flux	Stomach	-1.4^{a}	-1.0^{b}	0.0^{c}	0.9^{d}	0.07	***	L***
	Proximal	0.4^{a}	1.0^{a}	5.5^{b}	16.8^{c}	0.41	***	Q***
	Middle	-0.4^{c}	-0.7^{c}	-2.8^{b}	-10.0^{a}	0.26	***	Q***
	Distal	0.2	0.1	1.0	0.6	0.21	*	L*
Relative sodium flux	Stomach	-8.4^{a}	-5.2^{b}	0.7^{c}	6.7^{d}	0.26	***	L***
	Proximal	9.2^{d}	5.5^{c}	-2.6^{b}	-8.9^{a}	0.37	***	L***
	Middle	-4.3^{a}	-3.7^{a}	-1.8^{b}	-3.6^{a}	0.33	***	Q*
	Distal	0.1	-0.2	-0.3	0.3	0.31	ns	ns
Relative potassium flux	Stomach	-8.1	-8.0	-8.0	-7.9	0.06	ns	L*
	Proximal	0.3^{c}	0.2^{bc}	-0.2^{a}	-0.03^{ab}	0.07	**	L*
	Middle	-0.4	-0.6	-0.4	-0.6	0.07	ns	ns
	Distal	0.0	0.1	0.0	0.1	0.03	ns	ns

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). Estimations of the significant linear or quadratic relationships are Supplementary table S6.

Supplementary table S5. The estimated linear (L) and quadratic (Q) relationships with water salinity (X) of the dependent variables (relative calcium, magnesium, sodium, and potassium flux) depicted in Figure 2.

Dependent variables (mg g^{-1} ingested DM)	Equation (intercept (SE) ± β (SE) ± β_1 (SE))	R^2	Regression *p-value*
Relative calcium flux			
Stomach	-0.47 (0.16)	-	ns
Proximal	Y = 0.3 (0.7) – 0.03 (0.1) X + 0.01 (0.003) X^2	0.88	Q*
Middle	Y = -0.3 (0.5) + 0.2 (0.1) X – 0.01 (0.002) X^2	0.56	Q*
Distal	0.90 (0.22)	-	ns
Relative magnesium flux			
Stomach	Y = -1.5 (0.1) + 0.1 (0.004) X	0.96	L***
Proximal	Y = 0.3 (0.3) – 0.1 (0.04) X + 0.02 (0.001) X^2	0.995	Q***
Middle	Y = - 0.4 (0.2) + 0.1 (0.2) X – 0.01 (0.001) X^2	0.995	Q***
Distal	Y = 0.1 (0.1) + 0.02 (0.01) X	0.44	L*
Relative sodium flux			
Stomach	Y = -9 (0.3) + 0.4 (0.02) X	0.99	L***
Proximal	Y = 10 (0.6) – 0.5 (0.03) X	0.97	L***
Middle	Y = -5 (0.4) + 0.2 (0.1) X – 0.01 (0.002) X^2	0.48	Q*
Distal	-0.03 (0.14)	-	ns
Relative potassium flux			
Stomach	Y = -8 (0.04) + 0.01 (0.002) X	0.41	L*
Proximal	Y = 0.2 (0.1) – 0.01 (0.003) X	0.44	L*
Middle	-0.48 (0.04)	-	ns
Distal	0.05 (0.02)	-	ns

L, linear effect; Q, quadratic effect. ns, not significant, $p > 0.05$; *, $p < 0.05$; ***, $p < 0.001$.

Supplementary table S6. Progression of digestion of crude protein (CP ADC) in the stomach, proximal, middle, and distal intestine 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*
CP ADC (%)	Stomach	13.9	19.7	14.1	17.7	1.63	ns	ns
	Proximal	70.6^{b}	69.6^{ab}	65.1^{ab}	63.1^{a}	1.63	*	L**
	Middle	87.5^{b}	88.1^{b}	86.2^{ab}	84.0^{a}	0.60	**	L***
	Distal	89.7^{b}	88.9^{ab}	87.8^{ab}	86.9^{a}	0.48	*	L***

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).

Supplementary table S7. Concentration of ions in the isolated fluid phase of the chyme (mmol l^{-1}), in the proximal, middle, and distal intestine of Atlantic salmon smolt, as affected by increasing water salinity (0, 10, 20 and 35 ppt).

mmol l^{-1}		**0 ppt**	**10 ppt**	**20 ppt**	**35 ppt**	**pSEM**	**ANOVA** *p-value*
Calcium	Proximal	1.04^{a}	1.83^{a}	3.48^{b}	3.82^{b}	0.30	***
	Middle	0.57^{a}	1.54^{b}	2.65^{c}	2.81^{c}	0.14	***
	Distal	0.56^{a}	1.07^{a}	2.16^{b}	2.37^{b}	0.19	***
Sodium	Proximal	162^{c}	157^{c}	110^{b}	63.3^{a}	5.91	***
	Middle	161^{a}	162^{a}	125^{b}	73.7^{c}	3.03	***
	Distal	153^{c}	148^{c}	114^{b}	72.7^{a}	3.07	***
Potassium	Proximal	7.6^{b}	8.0^{b}	5.0^{a}	3.8^{a}	0.63	***
	Middle	7.7^{c}	7.3^{bc}	4.6^{ab}	3.6^{a}	0.93	**
	Distal	7.3^{b}	7.6^{b}	4.9^{ab}	3.1^{a}	1.23	*
Chloride	Proximal	27.0^{a}	39.0^{b}	61.0^{c}	78.0^{d}	2.17	***
	Middle	60.3^{a}	69.3^{ab}	83.0^{b}	83.3^{b}	6.17	*
	Distal	74.5^{a}	77.0^{ab}	91.5^{c}	$92.0.5^{bc}$	5.23	*

L, linear effect; Q, quadratic effect; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values are means (n = 4) and pooled standard errors of the mean (pSEM).

Supplementary table S8. Drinking rate (ml kg^{-1} h^{-1}) of Atlantic salmon smolts at different salinities.

	10 ppt	**20 ppt**	**35 ppt**
Relative drinking rate (ml kg^{-1} h^{-1})	0.78	3.75	4.11
% of water absorbed (%)	30.64	39.02	96.19
Rectal fluid secretion (ml kg^{-1} h^{-1})	0.03	-0.78	0.44

CHAPTER 5

Dietary electrolyte imbalance alters drinking rate and gastrointestinal tract water fluxes of Atlantic salmon (*Salmo salar*) smolt in seawater

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Abstract

It was hypothesised that dietary electrolyte balance (dEB) would influence the dynamics of water, ions, and nutrient fluxes in the gastrointestinal tract (GIT) of Atlantic salmon (*Salmo salar*) smolts differently depending on water salinity. To date, a comparative study on how dEB alters these dynamics in freshwater- and seawater-adapted fish is lacking. The test diets were low versus high dEB (-100 versus 500 mEq kg^{-1} DM^{-1}) and the test water salinities were 0 versus 30 ppt. Furthermore, the effect of the interaction between dEB and salinity on blood pH and osmolality were investigated. The experiment lasted for 6.5 weeks. Chyme was collected from 4 GIT segments (stomach, proximal, middle, and distal intestine) and analysed for dry matter (DM), pH, osmolality, crude protein, and ion (Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}) content. Water, ion, and nutrient fluxes were measured using yttrium oxide (Y_2O_3) as an inert marker. It was found that there was a diet effect on chyme pH in the stomach, being lower in fish fed the low dEB diet than the high dEB diet. Furthermore, the diet altered ion and nutrient fluxes in the stomach. Water salinity had the largest effect on chyme pH in all the GIT segments and on chyme osmolality in the stomach, which significantly increased in seawater conditions. The interaction between dEB and salinity had an effect on chyme DM, water and ion fluxes in the stomach, proximal and middle intestine. Our results showed that, depending on water salinity, dEB altered water fluxes differently. In freshwater-adapted fish, water influx to the stomach was higher in fish fed the high dEB diet than the low dEB diet, but the difference was neglectable. In contrast, in seawater-adapted fish, water influx into the stomach and proximal intestine was higher in fish fed the low dEB than the high dEB diet, and the amplitude was much larger. Additionally, in seawater conditions, drinking rate was 50% higher in fish fed the low dEB diet (3.07 ml kg^{-1} h^{-1}) than the high dEB diet (2.04 ml kg^{-1} h^{-1}). As a result, it was concluded that, in seawater conditions, a diet with a low dEB has a higher demand for endogenous water secretions in the stomach and proximal intestine of Atlantic salmon smolts as well as enhanced drinking rate.

5.1 Introduction

Dietary minerals can influence the ability of anadromous fish to adapt to seawater conditions (Zaugg, 1982). Salmonids benefit from a mineral-rich diet, resulting in increased tolerance and survival when transitioning to seawater (Basulto, 1976; Pellertier and Besner, 1992; Zaugg et al., 1983). This change is attributed to salt-induced gill remodeling at both the structural and molecular levels, resulting in a partial seawater adaptation characterized by increased expression of ion transporters (Basulto, 1976; Eroldoğan et al., 2005; Harpaz et al., 2005; Pellertier and Besner, 1992; Perry et al., 2006; Zaugg et al., 1983). Furthermore, the role of the intestine in salt and water balance, particularly in saltwater fish, has been recognized (Grosell, 2010; Whittamore, 2012; Wilson et al., 2002). Inadequate preparation of the intestine for the transition from freshwater to seawater, which coincides with changes in osmotic conditions, can have a negative impact on the performance of Atlantic salmon in seawater (Vargas-Lagos et al., 2018). Over the years, aquaculture feed formulation has evolved towards the inclusion of ingredients, alternative to fish meal and oil, primarily of plant origin. Next to impacts on macronutrient composition and digestibility the altered formulation can also change the dietary mineral or electrolyte composition and availability (Francis et al., 2001b). In this regard, our understanding of how dietary electrolytes (e.g., the dietary electrolyte balance, dEB) influences the regulation of osmoregulatory responses in the gastrointestinal tract (GIT) is still limited.

Dietary electrolyte balance (dEB, mEq kg^{-1} DM), often represented by the difference between monovalent cations (K^+ and Na^+) and anions (Cl^-) in the feed, influences the acid-base balance of monogastric animals (Sauvant et al., 2004). Dietary dEB depends on the mineral profile of the diet, which can be changed by mineral supplementation and by using different types of ingredients. Large variability in cation and anion contents between ingredients results in considerable variability in dEB between commercial feed formulations (Saravanan et al., 2013b). Historically, dEB in commercial

fish feeds in Europe ranged between 200 and 400 mEq kg^{-1} DM (Tacon and De Silva, 1983). Recently, more data has been made available on the dEB of commercial salmon feeds in Norway and it ranges from -9 to 455 mEq kg^{-1} feed based on life stage or feed category (Philip et al., 2022; Sele et al., 2023). The literature on the impact of dEB in fish is scarce, but it has been shown to affect acid-base balance, amino acid metabolism, feed intake, growth, energy utilization, chyme characteristics, water, ion, and nutrient kinetics in freshwater fish (Ciavoni et al., 2023; Magnoni et al., 2018b; Philip et al., 2022; Saravanan et al., 2013b). Imbalance in dietary electrolyte levels in fish feeds are expected to disrupt acid-base secretion in the gut and subsequently result in post-prandial systemic metabolic alkalosis or acidosis (Bucking and Wood, 2008). These disturbances in acid-base balance may activate compensatory mechanisms which affected chyme characteristics (dry mater, pH, osmolality), water and ion fluxes, and digestion kinetics in various segments of the GIT (Ciavoni et al., 2023). Most of the above-mentioned studies, however, were done in freshwater environment. Philip et al. (2022) studied the effect of dEB on carbonate precipitation in the intestinal lumen in Atlantic salmon after transfer to seawater, an important osmoregulatory function of the intestine. A low dEB (-25 to -50 mEq kg^{-1} DM) diet increased carbonate precipitation 24h post seawater transfer (Philip et al., 2022). Although the increased carbonate precipitate formation could be indicative of increased drinking in the low dEB diet, water and ion fluxes in the GIT were not studied by Philip et al. (2022).

Understanding the relationship between dietary composition and water salinity on osmoregulation in the GIT is critical for a species with a life cycle that includes both freshwater and saltwater environments, such as Atlantic salmon. This is because osmoregulation in teleosts, which change depending on environmental conditions, may interact with dietary characteristics and affect adaptation mechanisms. It was hypothesized that effects of dEB on osmoregulation mechanisms in salmon differ between freshwater and seawater. Therefore, feed formulation should be addressed to accommodate

for optimal adaptation during freshwater to seawater transfer. To study this, the effect of contrasting dEB (-100 versus 500 mEq kg^{-1} DM) and water salinity (0 versus 30 ppt) on water, ion, and nutrient fluxes in the gastrointestinal tract of Atlantic salmon (*Salmo salar*) smolts was investigated.

5.2 Material and methods

The feeding trial and sampling were conducted at Matre Research Station of Institute of Marine Research (IMR, 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 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.

5.2.1 Experimental design, animal housing and feeding

This experiment had a 2x2 factorial design. The first factor was the dietary electrolyte balance (dEB) (-100 versus 500 mEq kg^{-1} DM^{-1}). The second factor was water salinity (0 versus 30 ppt). The contrast in dEB was created by adding $CaCO_3$ and Na_2CO_3 to the high dEB diet and $CaCl_2$ to the low dEB diet (Table 1). The experiment involved a cohort of Atlantic salmon (*Salmo salar*) smolts comprising both male and female individuals (n = 640) prepared for transfer to seawater. The fish were allocated randomly to 16 tanks of 1 m^3 each, accommodating 40 fish per tank. These 16 tanks were randomly assigned to the four experimental groups (two dEB and salinity levels), with four replicates for each treatment. The tanks received a continuous flow of

water at a rate of 8 l min^{-1}, with the salinity adjusted according to the specific treatment. The photoperiod was set at 12:12, light:dark cycle, water temperature at 12 ºC, and the outlet's oxygen saturation was maintained above 80%. At the beginning of the experiment, the average weight of the fish was recorded (337 ± 5.5 g, mean ± SD). The experimental diets were 3.0 mm extruded sinking pellets and were produced by the Aquafeed technology center (Nofima AS, Bergen, Norway). The ingredient composition and analyzed nutrient content of the diets are given in Table 1. Diets were formulated to meet the nutrient requirements for Atlantic salmon smolts (NRC, 2011). Yttrium oxide (Y_2O_3) served as an inert marker in the feed to assess digestion kinetics and water/ion fluxes. The experiment lasted for 6.5 weeks, with 4 weeks on experimental diets to acclimate the fish to different salinities and stabilize their feed intake. The fish were fed twice daily, with each feeding lasting 2 hours. Automatic feeders were used to ensure consistent feeding until apparent satiation, and feed intake was carefully monitored by collecting and weighing any spilled feed pellets 15 minutes after each meal. Feed intake estimation followed the method proposed by Helland et al. (1996).

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

Test ingredients (%)	**Low dEB**	**High dEB**
Na_2CO_3	-	1.5
$CaCl_2$	2.4	-
$CaCO_3$	-	1.7
Basal ingredients (%)		
Wheat	21.2	20.4
Soya protein concentrate	20.0	20.0
Pea protein concentrate	20.0	20.0
Fishmeal LT (CP > 68%)	18.0	18.0
Fish oil	15.0	15.0
DL-Methionine	0.4	0.4
Histidine	0.5	0.5
Monocalciumphospate	1.5	1.5
Vitamin mineral premix[1]	1.0	1.0
Yttrium oxide	0.02	0.02
Sum	**100**	**100**
Proximate composition (%)		
Dry matter	94.0	90.0
Protein	45.7	47.8
Fat	19.4	19.6
Calcium	1.8	1.7
Sodium	0.5	1.2
Potassium	0.8	0.8
Magnesium	0.2	0.2
Phosphorus	1.4	1.4
dEB (mEq kg^{-1} DM)	-99	499

[1]Vitamin premix (giving the following concentrations per kg diet): vitamin A: 3000 IU; vitamin D3: 3800 IU; vitamin E: 300 mg; vitamin K3: 30 mg; vitamin B1: 30 mg; vitamin B2: 45 mg; vitamin B6: 38 mg; vitamin B12: 0.08 mg; niacin: 300 mg; Ca-D-pantonat: 90 mg; biotin: 1.5 mg; folic acid: 15 mg; vitamin C: 300 mg. Mineral premix (giving the following concentrations per kg diet): Mg, 0.1 g; Fe: 100 mg, Mn: 30 mg, Zn:130 mg, Cu: 6 mg, I: 5 mg, Co: 0.05 mg, Se: 0.3 mg.

5.2.2 Sampling

The final sampling was carried out over four days (days 43-46), with four tanks sampled each day. During this last feeding all the groups were fed 200 g feed $tank^{-1}$. Fish were sampled at fixed timepoint after the last meal, 6 hours postprandial, to standardize the measurements of chyme content. Feed intake during the last meal was measured by recording feed refusal as described above. All fish were euthanized in tricaine methanesulfonate (Finquel, MS-222, 0.5 g l^{-1}), counted 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 x 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 (10,000 RPM, for 5 min). In fresh plasma, osmolality (Micro-Osmometer, Fiske, Model 210) and ion (Ca^{2+}, Na^{+}, K^{+} and Cl^{-}) concentrations were measured (Radiometer, ABL90 FLEX plus). All fish per tank (n = 40) were then dissected for collection of chyme samples from the GIT. Chyme was quantitatively collected from four segments of the GIT: stomach, proximal, middle, and distal intestine. Chyme was analysed for pH, osmolality, dry matter, crude protein, mineral and yttrium content as described in detail by Ciavoni et al. (2023). In brief, the collected samples were pooled per tank, weighted, and stored in 150 ml plastic containers for each GIT segment. From these pooled chyme samples, a subsample of 2 ml was taken in an Eppendorf tube, centrifuged at 10,000 RPM, for 5 min 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 and diluted with cold distilled water ratio of 1:1 (w/v) to allow homogenization (Homogenizer, POLYTRON® PT 2100, Kinematica). After homogenization, the mixture was centrifuged at 3220 RPM for 30 minutes at 4 ˚C and the supernatant (enzyme extract) were 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, homogenized by pestle and mortar into a fine powder, and stored at 4 ºC until analysis to determine chyme nitrogen, mineral and yttrium content.

5.2.3 Analyses and calculations

The diets were homogenized and analysed for dry matter, ash, fat, and protein following standard procedures. Briefly, dry matter was measured after drying at 105 ºC for 24 h; ash content determined by combustion in a muffle furnace at 550 ºC 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 analyser (Vario Macro Cube, Elementar 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 and an inductively coupled plasma mass spectrometry (ICP-MS) as described elsewhere (Philip et al, 2021; Silva et al., 2019).

The feed intake per fish (FI, g $fish^{-1}$) was calculated as FI = (total offered feed – uneaten feed)/ (number of fish) (on DM basis). 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.

Water and mineral fluxes in the GIT were determined by using yttrium oxide (Y_2O_3) as a marker. As described by Harter et al. (2013) water and ion fluxes of Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+} were calculated in the stomach, proximal, middle, and distal intestine of the gastrointestinal tract. The water flux (ml g^{-1} of ingested DM feed) and ion fluxes (mg g^{-1} of ingested DM feed) in the stomach were calculated by subtracting the relative water or ion content in the stomach chyme from that in the diet and dividing by the relative amount of ingested feed dry matter. In the proximal, middle, and distal intestine, water and ion fluxes were calculated by subtracting the relative water or ion content in the

chyme of the intestinal segment from that in the chyme of the previous segment and dividing the result by the relative amount of ingested feed dry matter. The relative amount of 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.
For each diet group (low or high dEB), the drinking rate of Atlantic salmon in seawater (30 ppt) was calculated as a relative measure to those reared in freshwater, and hence referred to as relative drinking rate. The net flux of Mg^{2+} in the GIT was used as a proxy for ingested SW.

$$\text{Net GIT } Mg^{2+} \text{ flux (mg } g^{-1} \text{ of ingested DM feed)} = \sum Mg^{2+} \text{ flux}_{\text{(stomach + PI + MI + DI)}}$$

Once the net GIT Mg^{2+} flux was obtained, the difference (delta, Δ) between the net GIT Mg flux of the SW group (30 ppt) for which the drinking rate is to be measured and the freshwater group (0 ppt) was calculated.

$$\Delta_{(30 \text{ ppt} - 0 \text{ ppt})} Mg^{2+} \text{ flux} = \text{Net GIT } Mg^{2+} \text{ flux}_{(30 \text{ ppt})} - \text{Net GIT } Mg^{2+} \text{ flux}_{(0 \text{ ppt})}$$

Then, to obtain the volume of seawater drunk per g of DM ingested, the $\Delta_{(30 \text{ ppt} - 0 \text{ ppt})} Mg^{2+}$ flux was divided by the Mg^{2+} concentration in the water (mg/L) of the respective salinity (30 ppt). This provided the volume of SW drank by the fish (ml g^{-1} DM fed), which was further converted to per unit weight per hour.

$$\text{Relative drinking rate } (ml^{-1}\ kg^{-1}\ hr^{-1}\ g^{-1} \text{ DM fed}) = ([(\Delta\ Mg^{2+} \text{ flux})/ (Mg^{2+} \text{ conc. SW})]/t) * (1000/BW)$$

Where, t, is the post-prandial time point of sampling (6h in this study) and BW, is the body weight of the fish.

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 2-3 (Andreeva and Rumsh, 2001). Alkaline protease activity (U ml^{-1}) of intestinal chyme was measured using casein as substrate, according to Walter (1984). 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). 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).

5.2.4 Statistical analyses and graphical presentations

Fish tanks (n = 16) were used as experimental units for all analysed parameters and data are expressed as the mean ± SE per treatment of four replicates. 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 multivariate analysis was performed to test the effect of dietary electrolyte balance, water salinity and their interaction on all analysed parameters. 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. Figures were made using GraphPad Prism version 8.

5.3 Results

Feed intake (g $fish^{-1}$) and survival (%) were lower ($p < 0.001$) in SW fish compared to FW fish (Supplementary table S1). Feed intake was also affected by dietary electrolyte balance (dEB), being lower in fish fed the low dEB diet (avg. over salinities, 60.5 g $fish^{-1}$) compared to high dEB diet (avg. over salinities, 84.3 g $fish^{-1}$). Fish weight gain (g $fish^{-1}$) and SGR (% d^{-1}) were only affected by the diet, being lower ($p < 0.001$) in fish fed the low dEB diet compared to the high dEB diet. FCR was affected by dEB and the interaction between diet and salinity and it was lower ($p < 0.001$) in fish fed the high dEB (avg. over salinities, 1.54) diet compared to the low dEB diet (avg. over salinities, 3.12). Averaged over all treatments, fish final weight was 382 ± 22 g $fish^{-1}$ and fish survival were 98.6%.

5.3.1 Chyme dry matter and relative water fluxes

There was an interaction effect ($p < 0.001$) of dEB and water salinity on chyme dry matter (DM) and relative water fluxes (RWF) in the stomach, proximal and middle intestine (Figure 1; Supplementary table S2).

When fed the high dEB diet, chyme DM was lower in in the GIT of freshwater-adapted fish and higher in seawater-adapted fish compared to low dEB diet (interaction effect, $p < 0.001$). In FW conditions, the largest difference in chyme DM was found in the stomach, where it was 30% and 27.4% in the low and high dEB fed fish, respectively. In SW conditions, the largest difference was found in the proximal intestine, where chyme DM was 16.1% and 12.6% in the high and low dEB fed fish, respectively. When fed the high dEB diet, water influx was higher in the stomach of freshwater-adapted fish and lower in seawater-adapted fish (interaction effect, $p < 0.01$). In the proximal and middle intestine, the largest differences in RWF caused by the diet were present in seawater-adapted fish. In the proximal intestine of SW-adapted fish, water influx was almost three times higher ($p < 0.001$) in fish fed the low dEB diet (24.0 ml g^{-1} ingested DM) compared to the high dEB diet (8.9 ml g^{-1} ingested DM). In the middle intestine of SW-adapted

fish, water efflux was more doubled ($p < 0.001$) in fish fed the low dEB diet (-26.7 ml g^{-1} ingested DM) compared to the high dEB diet (-11.2 ml g^{-1} ingested DM).

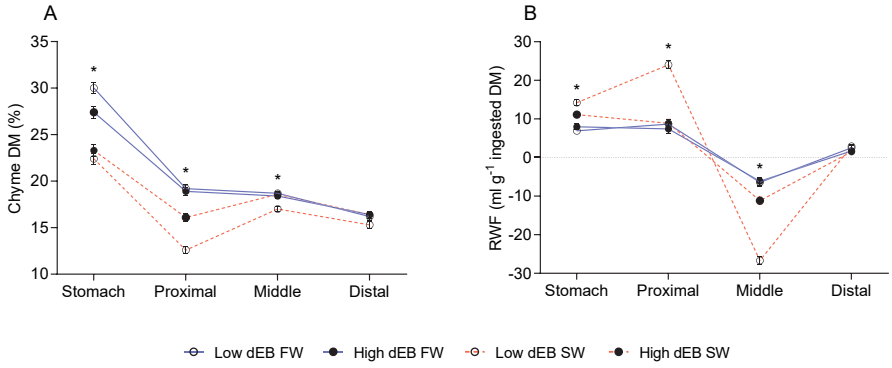

Figure 1. (A) Chyme dry matter, DM and (B) relative water fluxes, RWF, as affected by increasing water salinity (0 and 35 ppt) and contrasting (low versus high) electrolyte balance (dEB) in the stomach, proximal, middle, and distal intestine of Atlantic salmon. The blue lines indicate freshwater (FW) condition and the red lines seawater (SW) condition. The empty dots (○) and dotted line represent the low dEB diet and the full dots (●) and continuous line represent the high dEB diets. The asterisk (*) indicates the presence of an interaction effect between water salinity and dietary treatment. Values are expressed as the mean per treatment (n = 4) and standard error of the means (SEM). The single effect of each treatment is presented in the Supplementary table S2.

5.3.2 Chyme pH and osmolality

There was no interaction effect between water salinity and diet on chyme pH and chyme osmolality (Osm) in all the GIT tract segments (Supplementary table S2). Therefore, only the main effect of diet and salinity are addressed in Figure 2.

Water salinity affected chyme pH in all segments of the GIT, being higher ($p < 0.001$) in seawater-adapted fish (Figure 2A). Chyme pH was affected by the diet only in the stomach ($p < 0.01$) (Figure 2B). Averaged over water salinities, chyme pH in the stomach was 4.16 and 4.96 in the low dEB and high dEB group, respectively. In all the intestinal segments, chyme pH was similar between both diets. Water salinity affected ($p < 0.001$) chyme Osm

only in the stomach (Figure 2C). Averaged over diets, chyme Osm was 247 and 789 mmol kg^{-1} in the stomach of FW and SW fish, respectively. The diet had an effect ($p < 0.01$) on chyme Osm only in the proximal intestine. Averaged over salinities, chyme Osm was 428 and 478 mmol kg^{-1} in the low dEB and high dEB group, respectively.

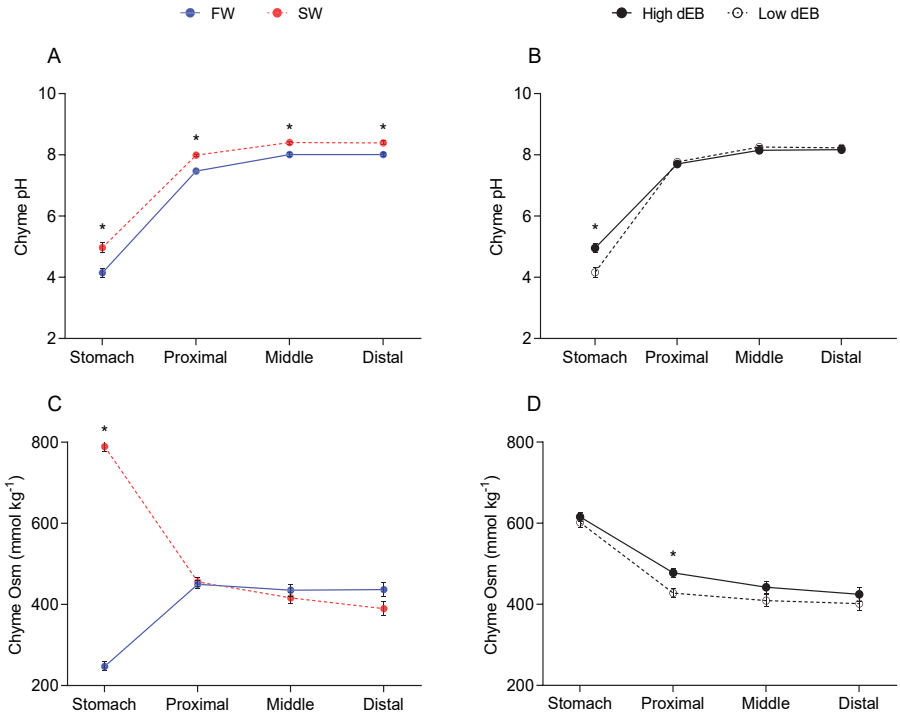

Figure 2. (A, B) Chyme pH and (C, D) Chyme osmolality (Osm), as affected by increasing water salinity (0 and 35 ppt) and contrasting (low versus high) electrolyte balance (dEB) in the stomach, proximal, middle, and distal intestine of Atlantic salmon. In the left panels, the blue dots and continuous line represent freshwater (FW) condition and the red dots and dotted line represent seawater (SW) condition. In the right panels, the empty dots (○) and dotted line represent the low dEB diet and the full dots (●) and continuous line represent the high dEB diets. The asterisk (*) indicates the presence of an interaction effect between water salinity and dietary treatment. Values are expressed as the mean per treatment (n = 4) and standard error of the means (SEM). The interaction effects are presented in the Supplementary table S2.

5.3.3 Ion fluxes

The interaction effect of diet and water salinity had no effect on the majority of Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+} fluxes. As a result, the main effect is shown in Figure 3, and significant interaction effects are mentioned in the text (Supplementary table S3). The main effect of the diet on relative Ca^{2+} flux (RCaF) was present in the stomach, proximal and distal intestine (Figure 3B). In the stomach, RCaF was lower ($p < 0.001$) in fish fed the low dEB diet (-7.8 mg g^{-1} ingested DM) compared to the high dEB diet (0.2 mg g^{-1} ingested DM). In the proximal intestine, there was an influx of Ca^{2+} in fish fed both dietary treatments, being higher ($p < 0.001$) in fish fed the low dEB diet (8.5 mg g^{-1} ingested DM) compared to the high dEB diet (2.2 mg g^{-1} ingested DM). In the distal intestine, RCaF dropped being lower ($p < 0.05$) in fish fed the high dEB diet (-0.1 mg g^{-1} ingested DM) compared to the low dEB diet (2.1 mg g^{-1} ingested DM). Water salinity had an effect on RCaF only in the proximal intestine, where it was higher ($p < 0.05$) in SW (6.2 mg g^{-1} ingested DM) than in FW conditions (4.5 mg g^{-1} ingested DM) (Figure 3A).

The main effects of the diet and water salinity on relative Mg^{2+} flux (RMgF) was present in the stomach (Figure 3C, 3D). In the proximal and middle intestine, there was an effect of the interaction between diet and salinity, and the largest differences were present in seawater-adapted fish (Supplementary table S3). In the proximal intestine of SW-adapted fish, RMgF was almost twice higher ($p < 0.001$) in fish fed the low dEB diet (14.7 mg g^{-1} ingested DM) compared to the high dEB diet (8.8 mg g^{-1} ingested DM). In the middle intestine of SW-adapted fish, Mg^{2+} efflux was larger ($p < 0.001$) in fish fed the low dEB diet (-9.9 mg g^{-1} ingested DM) compared to the high dEB diet (-6.8 mg g^{-1} ingested DM).

The main effect of water salinity on relative Na^{+} flux (RNaF) was present in all GIT segments, whereas the main effect of the diet was present only in the stomach and middle intestine (Figure 3E, 3F). Averaged over diets, Na^{+} influx was 16.0 and -3.5 mg g^{-1} ingested DM in the stomach of seawater- and freshwater- adapted fish, respectively. Averaged over water salinities, the diet effect in the stomach caused a larger ($p < 0.001$) Na^{+} influx in fish fed the

low dEB diet (10.4 mg g^{-1} ingested DM) compared to the high dEB diet (2.1 mg g^{-1} ingested DM). In the proximal and distal intestine, Na^+ flux was only affected by water salinity ($p < 0.001$) and averaged over both diets, it was 9.0 and 16.9 mg g^{-1} ingested DM, in the proximal intestine and -0.1 and 0.7 mg g^{-1} ingested DM in the distal intestine of freshwater- and seawater-adapted fish, respectively. In the middle intestine, there was an effect of the interaction between diet and salinity ($p < 0.05$) (Supplementary table S3).

Relative K^+ flux (RKF) was affected ($p < 0.05$) only by water salinity in the distal intestine, whereas no dietary effect was detected (Figure 3G, 3H). Averaged over both diets and salinities, the relative K^+ flux was -5.7, -0.6, -0.8, and 0.03 mg g^{-1} ingested DM in the stomach, proximal, middle, and distal intestine, respectively (Supplementary table S3).

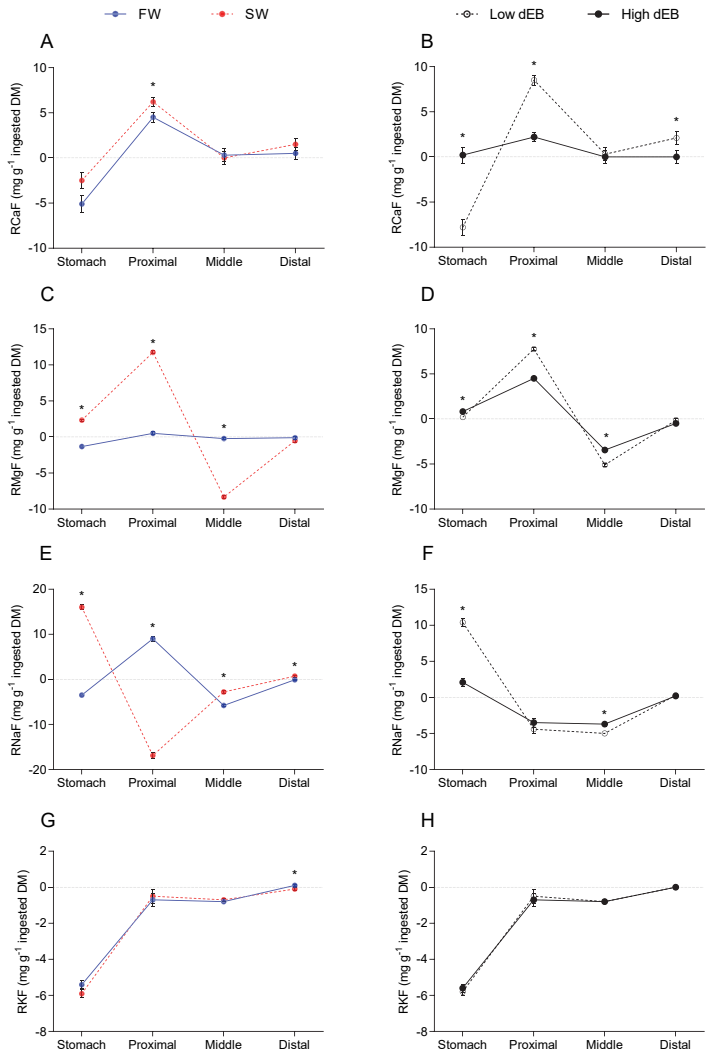

Figure 3. Relative fluxes of (A, B) calcium, (RCaF); (C, D) magnesium, (RMgF); (E, F) sodium, (RNaF) and (G, H) potassium, (RKF) in the stomach, proximal, middle, and distal intestine of Atlantic salmon as affected by increasing water salinity and contrasting dietary electrolyte balance (dEB). In the left panels, the blue dots and continuous line represent freshwater (FW) condition and the red dots and dotted line represent seawater (SW) condition. In the right panels, the empty dots (○) and dotted line represent the low dEB diet and the full dots (●) and continuous line represent the high dEB diets. The asterisk (*) indicates the presence of an interaction effect between water salinity and dietary treatment. Values are expressed as the mean per treatment (n = 4) and standard error of the means (SEM). The interaction effects are presented in the Supplementary table S3.

5.3.4 Relative drinking rate

Relative drinking rate (ml kg^{-1} h^{-1} g DM $ingested^{-1}$) of Atlantic salmon smolts adapted to seawater (30 ppt) fed diets contrasting in dietary electrolyte balance (dEB) is depicted in Figure 4. Relative drinking rate increased by ~50%, from 2.05 to 3.07 ml kg^{-1} h^{-1} g DM $ingested^{-1}$, when fish were fed the high and the low dEB diet, respectively ($p < 0.001$).

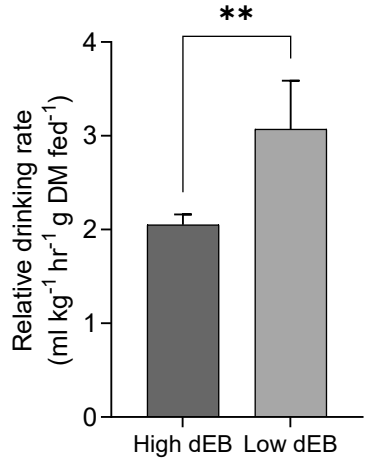

Figure 4. Relative drinking rate of seawater-adapted Atlantic salmon smolts fed the low dEB diet (-100 mEq kg^{-1} DM) and the high dEB diet (500 mEq kg^{-1} DM). Data are presented as mean and standard deviation (SD), and the asterisks (** = $p < 0.01$) indicate the presence of a significant effect of diet on relative drinking rate.

5.3.5 Blood pH, plasma osmolality and ion content

The effect of diet, water salinity and the interaction between diet and salinity on caudal blood pH, plasma osmolality (Osm) and plasma ion concentration are presented in Table 2. Caudal blood pH was unaffected by diet, water salinity, and their interaction. Plasma osmolality was higher ($p < 0.001$) in seawater-adapted fish (avg. over diets, 333 mmol l^{-1}) than in freshwater-adapted fish (avg. over diets, 324 mmol l^{-1}). Plasma Na^+ and K^+ concentration was affected by the interaction between diet and salinity, whereas no interaction effect was present on plasma Ca^{2+} and Cl^- concentration.

Table 2. Blood pH in the caudal vein, osmolality, and ion concentration (mmol l^{-1}) in plasma of Atlantic salmon smolt as affected by contrasting water salinity (FW versus SW) and dietary electrolyte balance (low versus high dEB).

	FW		**SW**			***p-values***		
	Low dEB	**High dEB**	**Low dEB**	**High dEB**	**pSEM**	**Salinity**	**Diet**	**Salinity*Diet**
Blood pH	7.17	7.15	6.69	7.02	0.19	ns	ns	ns
Plasma osmolality	323^a	326^a	333^b	334^b	1.24	***	ns	ns
Plasma Ca^{2+}	1.2	1.3	1.2	1.3	0.03	ns	ns	ns
Plasma Na^+	160^a	162^a	166^b	165^b	0.61	***	ns	*
Plasma K^+	3.9^a	3.9^a	3.8^a	4.8^b	0.17	*	**	**
Plasma Cl^-	134	133	135	135	0.63	ns	ns	ns

Ca^{2+}, calcium; Na^+, sodium, K^+, potassium, Cl^-, chloride. ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values are expressed as the mean per treatment ($n = 4$) and pooled standard errors of the mean (pSEM).

5.3.6 Crude protein digestion kinetic

Kinetic of crude protein digestion (CP ADC) was not affected by the interaction between diet and water salinity in all the segments of the gastrointestinal tract. Therefore, the main effects of water salinity and diet are presented separately in Figure 5. There was a main effect of the diet on CP ADC only in the stomach, where it was lower ($p < 0.05$) in fish fed the low dEB diet (avg. over water salinities, 14.5%) compared to the high dEB diet (avg. over water salinities, 23.3%).

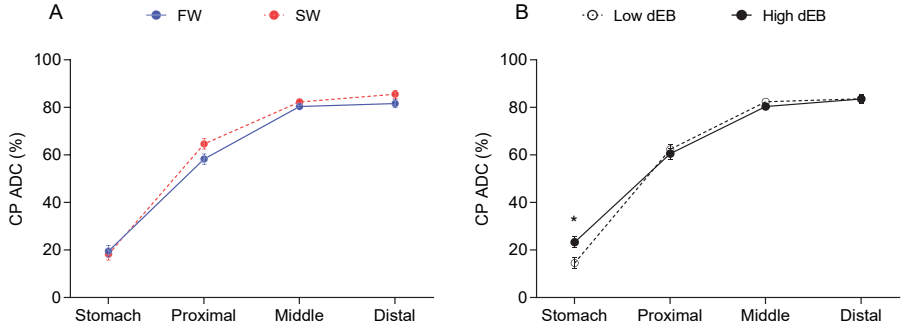

Figure 5. Progression of digestion of crude protein (CP ADC) in the stomach, proximal, middle, and distal intestine of Atlantic salmon as affected by increasing water salinity and contrasting dietary electrolyte balance (dEB). In the left panels, the blue dots and continuous line represent freshwater (FW) condition and the red dots and dotted line represent seawater (SW) condition. In the right panels, the empty dots (○) and dotted line represent the low dEB diet and the full dots (●) and continuous line represent the high dEB diets. The asterisk (*) indicates the presence of an interaction effect between water salinity and dietary treatment. Values are expressed as the mean per treatment (n = 4) and standard error of the means (SEM). The single effect of each treatment is presented in the Supplementary table S5.

5.3.7 Proteolytic enzyme activity in the chyme

The effect of diet and water salinity on pepsin activity in the stomach and protease activity in the proximal, middle, and distal intestine is presented in Table 3. There was no effect of the interaction between diet and salinity on enzyme activity in all the segments of the GIT. In the stomach and distal intestine, enzyme activity was affected only by water salinity. In the stomach, pepsin activity was lower ($p < 0.01$) in SW-adapted fish (avg. over diets, 57.7 U mg^{-1} chyme ww) than FW-adapted fish (avg. over diets, 93.5 U mg^{-1} chyme ww). In the distal intestine, protease activity was lower ($p < 0.05$) in SW-adapted fish (55.9 U mg^{-1} chyme ww) than FW-adapted fish (74.1 U mg^{-1} chyme ww). In the middle intestine, protease activity was affected only by the diet, being lower in fish fed the high dEB diet (avg. over water salinities, 92.0 U mg^{-1} chyme ww) compared to high dEB diet (avg. over water salinities, 150.3 U mg^{-1} chyme ww).

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 dietary electrolyte balance (dEB) and water salinity.

		FW		SW			*p-values*		
U mg^{-1} chyme ww		**Low dEB**	**High dEB**	**Low dEB**	**High dEB**	**pSEM**	**Salinity**	**Diet**	**Salinity*Diet**
Pepsin	**Stomach**	99.5^{c}	87.5^{bc}	62.7^{ab}	52.7^{a}	6.52	**	ns	ns
Protease	**Proximal**	116.1	85.6	86.4	77.9	18.7	ns	ns	ns
	Middle	169.2^{b}	88.1^{a}	131.4^{ab}	95.9^{ab}	18.6	ns	**	ns
	Distal	75.0	73.2	56.4	55.3	7.82	*	ns	ns

FW, 0 ppt salinity; SW, 30 ppt salinity; low dEB, -100 mEq kg^{-1} DM; high dEB, +500 mEq kg^{-1} DM. ww, wet weight; ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$. Values are expressed as the mean per treatment ($n = 4$) and the pooled standard errors of the mean (pSEM).

5.4 Discussion

The current study investigated the role of dietary electrolyte balance (dEB) and water salinity on digestion and osmoregulation in the GIT in Atlantic salmon smolts. Increasing dEB led to higher chyme pH in the stomach of freshwater- and seawater-adapted salmon. Similarly, previous research has shown that, when fed high dEB diets, chyme pH increased in the stomach of freshwater Nile tilapia (*Oreochromis niloticus*) (Saravanan et al., 2013b) and rainbow trout (*Oncorhynchus mykiss*) (Magnoni et al., 2018; Ciavoni et al., 2023). Furthermore, in the present study, a diet effect on ion fluxes was present in the stomach. Indeed, the low dEB diet promoted a significant efflux of Ca^{2+} and an influx of Na^{+} in the stomach of fish when compared to high dEB diet (Figure 3B, 3F). The higher divalent ion efflux in low dEB fed fish may be a consequence of the lower chyme pH in the stomach, which may cause Ca^{2+} and Mg^{2+} to dissolve faster and move to the proximal intestine as suggested by Bucking and Wood (2007) and Elesho et al. (2022). Our results are consistent with previous studies and indicate that dEB mainly affects acid-base balance in the stomach of fish during digestion (Magnoni et al., 2018b, 2017; Saravanan et al., 2013b). Furthermore, the increase in divalent ions (Ca^{2+}, Mg^{2+}) influx in the proximal intestine was larger in the low dEB group than in the high dEB group. This implies that the increased acid secretion in the stomach drives more alkaline secretion in the proximal intestine. Additionally, chyme osmolality decreased in the proximal intestine of fish fed the low dEB diet, which could be attributed to the intestinal bicarbonate secretion to aid precipitation of calcium-magnesium carbonate for water absorption in the mid intestine (Philip et al., 2022; Wilson and Grosell, 2003). Elesho et al. (2022) proposed that when African catfish (*Clarias gariepinus*) were fed diets contrasting in starch versus fat, endogenous secretions in the stomach and proximal intestine were altered affecting Ca^{2+} and Mg^{2+} fluxes. This gives support to our observations that ion fluxes in the proximal intestine are regulated by changes in the stomach caused by diet. Furthermore, the calculated ADC of crude protein (CP) in the stomach suggests a quicker

movement of solubilized proteins from the stomach to the proximal intestine in fish fed the low dEB diet than the high dEB diet, but no dietary effect was present in the intestine (Figure 5B). Previous research has shown that high dEB diet may lead to higher hydration in the stomach of freshwater-adapted fish, hence facilitating digestive processes (Magnoni et al., 2018; Ciavoni et al., 2023). However, in the current study, water influx in the stomach was higher in fish fed the high dEB diet in freshwater conditions, but not in seawater conditions. This may be an indication that dEB alters water and nutrient fluxes in the stomach differently, depending on environmental salinity. In contrast to CP digestibility, enzyme activity in the stomach was not altered by dEB, but it was significantly higher in the middle intestine of fish fed the low dEB diet (Table 3). This could be a result of the more alkaline chyme. Magnoni et al. (2017) proposed that differences in dietary dEB may alter chyme pH in the gut of the euryhaline meagre fish (*Argyrosomus regius*) and affect enzyme activity. However, they did not find changes in trypsin and chymotrypsin activity in the intestine in fish fed contrasting dEB diets (200 versus 700 mEq kg^{-1} DM). Overall, when looking at chyme characteristics, the more acidic low dEB diet and the more alkaline high dEB diet only affected chyme pH in the stomach. Similarly, ion and nutrient fluxes were differentially affected by dEB only in the stomach and proximal intestine.

Depending on environmental salinity, water fluxes in fish GIT change for osmotic purposes. Water fluxes include endogenous secretions produced during digestion or ingestion of water (drinking) from the environment. Drinking rate in unfed Atlantic salmon pre-smolt increases from 1.3 ml kg^{-1} h^{-1} in FW to 6.45 ml kg^{-1} h^{-1} in SW (Eddy, 2007; Smith et al., 1991; Usher et al., 1988). When fish are fed, their drinking rate increases further in both FW and SW environment (Bucking and Wood, 2006a; Eddy, 2007; Wood and Bucking, 2010). Accordingly, water influx in the GIT was higher in SW-adapted fish than FW-adapted fish. The higher water influx in SW condition influenced chyme osmolality in the stomach (Figure 2C), which was more than three times higher (789 mmol kg^{-1}) compared to FW condition (247

mmol kg^{-1}). However, chyme osmolality dropped from the proximal intestine to the distal intestine and the difference between FW- and SW-conditions were not significant, further confirming that the osmolality is only regulated in the intestinal segments but not in the stomach. Furthermore, chyme pH in the stomach and intestinal segments of Atlantic salmon increased along with water salinity (Figure 2A). In contrast, Usher et al. (1990) showed that chyme pH in the stomach did not change in Atlantic salmon smolts after transfer to seawater. Similarly, Ciavoni et al. (2024) proposed that, albeit drinking rate increased in seawater-adapted Atlantic salmon, water influx and chyme pH in the stomach did not differ between 0 and 35 ppt salinity when fed a commercial-like diet (ca. dEB of 260 mEq kg^{-1} DM). In both the studies, however, chyme pH was higher in the intestinal segments of seawater- than freshwater-adapted salmon.

Ciavoni et al. (2024) found that water influx increased more than three times between the stomach and the proximal intestine in seawater-adapted salmon. Therefore, it was proposed that, in seawater conditions, ingested water is shunted directly from the stomach into the proximal intestine, where it is primarily intended for osmoregulatory water uptake rather than chyme liquefaction in the stomach. In the present study, the salinity effect on water fluxes in the GIT changed significantly depending on the dEB of the feed (interaction effect) (Figure 1A, 1B). Therefore, the water salinity effect on water and ion fluxes needs to be discussed considering its interaction with the diet effect.

The interaction between dietary dEB and water salinity significantly altered chyme DM, water, and ion fluxes in the GIT of Atlantic salmon smolts. In the stomach, chyme DM was lower in freshwater-adapted fish fed the high dEB diet. Previous research suggested that, when freshwater fish were fed a high dEB diet, more acidic fluid secretion was needed to lower chyme pH into the stomach, decreasing chyme DM (Ciavoni et al., 2023; Magnoni et al., 2018b; Saravanan et al., 2013b). In the stomach, the effect of the interaction between diet and salinity on chyme DM was less pronounced, but

it was stronger on water influx. Water influx in the stomach increased by 3.1 ml water g^{-1} ingested DM with the low dEB diet in seawater conditions. This could be due to a rise in endogenous secretions as well as water intake promoted by the diet. Indeed, in seawater conditions, relative drinking rate was higher in fish fed the low dEB diet (3.07 ml kg^{-1} h^{-1}) than the high dEB diet (2.04 ml kg^{-1} h^{-1}) (Figure 4). Ingested seawater, therefore, appears to remain in the stomach after feeding the low dEB diet instead of quickly moving to the proximal intestine, as proposed by Ciavoni et al., (2024). As a result, our findings indicate that dietary dEB may aid or inhibit drinking as well as endogenous secretions in the stomach in seawater conditions. This may be due to the intrinsic dietary characteristics which may demand more water addition into the stomach to facilitate nutrient hydrolysis. The measurement of ingested seawater in the current study was based on the net GIT flux of Mg^{2+} at 30 ppt salinity relative to the Mg^{2+} flux in freshwater and is thus referred to as the 'relative' drinking rate. Among the two divalent cations (Ca^{2+} and Mg^{2+}) that are ideal candidates for studying drinking in fish, magnesium was chosen because in our previous study (Ciavoni et al., 2024), an isosmotic point of 12 ppt was achieved based on Mg^{2+} flux, whereas it was unable to do so based on Ca^{2+} flux, as some Ca^{2+} uptake is known to occur in freshwater. Although it is possible that Mg^{2+} from ingested SW can also be absorbed in the GIT, Lin et al. (2013) demonstrated that Mg^{2+} from SW is absorbed only when the dietary Mg^{2+} supply is deficient, which was not the case in the present study. On the contrary, Mg^{2+} from ingested SW is concentrated (Shehadeh and Gordon, 1969; Wood et al., 2004) precipitated as calcium magnesium carbonate in the intestinal lumen of fish at higher water salinities (Philip et al., 2022; Walsh et al., 1991). Despite being fed the same diet Mg^{2+} levels, differences in actual feed intake resulted in slightly different dietary Mg^{2+} intake levels across groups. However, when compared to the increase in Mg^{2+} concentrations in the chyme with increasing salinity, these differences were negligible. Furthermore, in seawater-adapted fish, water influx nearly doubled between the stomach and the proximal intestine

when fed the low dEB diet, whereas it slightly decreased in fish fed the high dEB diet. This suggests that ingested water may transiently remain in the stomach and partially pass directly to the proximal intestine. Moreover, endogenous alkaline secretions may further contribute to increasing water influx in the proximal intestine (Grosell, 2010, 2006). Accordingly, Ciavoni et al. (2024) proposed that chyme liquefaction in the stomach is endogenous, whereas in the intestine it is both endogenous and exogenous. However, whether of exogenous or endogenous origin, the current study found that the interaction between water salinity and dEB is critical in determining the magnitude of water fluxes along the GIT. Furthermore, when fed the low dEB diet, the influx of divalent ions (Ca^{2+}, Mg^{2+}) into the proximal intestine of seawater-adapted fish increased (Figure 3B, 3D). Consequently, reabsorption of water and ions in the middle intestine of seawater-adapted fish was significantly higher in the low dEB group. The present results, further support the hypothesis that, depending on water salinity, dEB alters water and ion fluxes in the GIT differently.

In summary, dietary dEB alters water, ion, and nutrient fluxes in the GIT, but differently depending on environmental salinity. The influence of contrasting dEB on regulation of water dynamics in the GIT was larger in seawater than in freshwater smolt Atlantic salmon. As a result, it can be concluded that dEB plays a critical role in regulating water ingestion and fluxes in the GIT of Atlantic salmon smolts in seawater. Based on these findings, it is suggested that physiological constraints that may arise from the interaction between diet and environment warrant more consideration in formulation of smolt feeds.

Acknowledgments

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5.5 Supplementary tables

Supplementary table S1. Growth performance of Atlantic salmon smolt fed a commercial diet for 6 weeks as affected by contrasting dietary electrolyte balances (dEB) and water salinity.

	FW		SW			*p-values*		
	Low dEB	**High dEB**	**Low dEB**	**High dEB**	**pSEM**	**Salinity**	**Diet**	**Salinity*Diet**
Feed intake (g fish^{-1})	77.1^{bc}	102.5^{c}	43.9^{a}	66.1^{ab}	6.41	***	**	ns
Weight gain (g fish^{-1})	31.6^{a}	64.1^{b}	26.0^{a}	55.5^{ab}	7.16	ns	***	ns
SGR (% day^{-1})	0.21^{a}	0.40^{b}	0.16^{a}	0.34^{ab}	0.04	ns	***	ns
FCR	2.68^{b}	1.62^{a}	3.56^{b}	1.46^{a}	0.23	ns	***	*
Survival (%)	100	100	96.3	97.2	1.02	-	-	-

FW, 0 ppt salinity; SW, 30 ppt salinity; low dEB, -100 mEq kg^{-1} DM; high dEB, +500 mEq kg^{-1} DM. SGR, specific growth rate; FCR, feed conversion ratio on dry matter basis; ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values are expressed as the mean per treatment (n = 4) and pooled standard errors of the mean (pSEM).

Supplementary table S2. Chyme dry matter (DM), pH, osmolality and relative water fluxes (RWF) in the stomach, proximal, middle, and distal intestine of Atlantic salmon smolt.

		FW		SW			*p-values*		
		Low dEB	High dEB	Low dEB	High dEB	pSEM	Salinity	Diet	Salinity*Diet
Chyme DM (%)	Stomach	30.0^{c}	27.4^{b}	22.4^{a}	23.3^{a}	0.62	***	ns	*
	Proximal	19.3^{c}	18.9^{c}	12.6^{a}	16.1^{b}	0.41	***	**	***
	Middle	18.7^{b}	18.4^{b}	17^{a}	18.6^{b}	0.27	*	*	**
	Distal	16.2	16.4	15.3	16.4	0.39	ns	ns	ns
Chyme pH	Stomach	3.84^{a}	4.46^{a}	4.48^{a}	5.46^{b}	0.22	**	**	ns
	Proximal	7.50^{a}	7.45^{a}	8.04^{b}	7.95^{b}	0.05	***	ns	ns
	Middle	8.07^{ab}	7.96^{a}	8.46^{c}	8.34^{bc}	0.07	***	ns	ns
	Distal	8.04	7.98	8.42	8.35	0.11	**	ns	ns
Chyme osmolality (mmol kg^{-1})	Stomach	237^{a}	258^{a}	784^{b}	795^{b}	15.8	***	ns	ns
	Proximal	428	471	427	485	14.7	ns	**	ns
	Middle	425	445	393	439	20.9	ns	ns	ns
	Distal	429	444	375	405	25.3	ns	ns	ns
RWF (ml water g^{-1} ingested DM)	Stomach	6.9^{a}	8.0^{a}	14.2^{c}	11.1^{b}	0.72	***	ns	**
	Proximal	8.7^{a}	7.4^{a}	24.0^{b}	8.9^{a}	1.08	***	***	***
	Middle	-6.5^{c}	-6.1^{c}	-26.7^{a}	-11.2^{b}	0.99	***	***	***
	Distal	2.5	1.7	2.9	1.5	0.52	ns	ns	ns

FW, 0 ppt salinity; SW, 30 ppt salinity; low dEB, -100 mEq kg^{-1} DM; high dEB, +500 mEq kg^{-1} DM. ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values are expressed as the mean per treatment (n = 4) and the pooled standard errors of the mean (pSEM).

Supplementary table S3. Relative calcium, magnesium, sodium and potassium fluxes in the stomach, proximal, middle, and distal intestine of Atlantic salmon smolt, as affected by contrasting dietary electrolyte balances (dEB) and water salinity.

		FW		SW			*p-values*		
		Low dEB	**High dEB**	**Low dEB**	**High dEB**	**pSEM**	**Salinity**	**Diet**	**Salinity*Diet**
RCaF (mg g^{-1} ingested DM)	Stomach	-8.66^{a}	-1.63^{bc}	-6.89^{ab}	1.99^{c}	1.26	ns	***	ns
	Proximal	7.45^{b}	1.49^{a}	9.48^{b}	2.91^{a}	0.77	*	***	ns
	Middle	-0.01	0.53	0.66	-0.57	1.07	ns	ns	ns
	Distal	1.82	-0.77	2.29	0.67	0.95	ns	*	ns
RMgF (mg g^{-1} ingested DM)	Stomach	-1.61^{a}	-1.06^{a}	2.02^{b}	2.69^{b}	0.19	***	**	ns
	Proximal	0.85^{a}	0.20^{a}	14.7^{b}	8.82^{b}	0.28	***	***	***
	Middle	-0.33^{c}	-0.12^{c}	-9.88^{a}	-6.76^{b}	0.25	***	***	***
	Distal	0.003	-0.19	-0.24	-0.80	0.31	ns	ns	ns
RNaF (mg g^{-1} ingested DM)	Stomach	0.22^{b}	-7.15^{a}	20.6^{d}	11.4^{c}	0.78	***	***	ns
	Proximal	8.43^{b}	9.63^{b}	-17.3^{a}	-16.6^{a}	0.90	***	ns	ns
	Middle	-5.86^{a}	-5.68^{ab}	-4.06^{b}	-1.62^{c}	0.43	***	*	*
	Distal	-0.07	-0.13	0.74	0.59	0.26	*	ns	ns
RKF (mg g^{-1} ingested DM)	Stomach	-5.83	-4.98	-5.71	-6.15	0.33	ns	ns	ns
	Proximal	-0.38	-1.01	-0.62	-0.47	0.53	ns	ns	ns
	Middle	-0.76	-0.83	-0.75	-0.69	0.11	ns	ns	ns
	Distal	0.15	0.02	-0.18	-0.11	0.09	*	ns	ns

FW, 0 ppt salinity; SW, 30 ppt salinity; low dEB, -100 mEq kg^{-1} DM; high dEB, +500 mEq kg^{-1} DM. ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values are expressed as the mean per treatment (n = 4) and pooled standard errors of the mean (pSEM).

Supplementary table S4. Concentration of ions in the isolated fluid phase of the chyme (mmol l^{-1}), in the stomach, proximal, middle, and distal intestine of Atlantic salmon smolt, as affected by contrasting dietary electrolyte balances (dEB) and water salinity.

		FW		SW			*p-values*		
		Low dEB	**High dEB**	**Low dEB**	**High dEB**	**pSEM**	**Salinity**	**Diet**	**Salinity*Diet**
Calcium	Proximal	2.73	0.55	5.55	4.08	0.606	***	*	ns
	Middle	2.23	0.38	3.63	2.70	0.418	***	**	ns
	Distal	2.30	0.39	3.91	1.88	0.362	***	***	ns
Sodium	Proximal	146.5	146.0	63.3	69.5	3.381	***	ns	ns
	Middle	155.3	148.0	79.0	97.5	3.57	***	ns	**
	Distal	140.5	141.8	82.0	105.8	4.74	***	*	*
Potassium	Proximal	8.50	9.98	4.13	4.45	1.067	***	ns	ns
	Middle	9.60	8.90	3.80	4.80	1.216	**	ns	ns
	Distal	9.65	9.63	3.55	4.18	1.784	**	ns	ns
Chloride	Proximal	46.8	34.8	57.0	53.0	5.744	*	ns	ns
	Middle	77.0	60.0	46.0	41.5	6.654	**	ns	ns
	Distal	67.8	52.5	46.5	39.8	3.426	***	**	ns

FW, 0 ppt salinity; SW, 30 ppt salinity; low dEB, -100 mEq kg^{-1} DM; high dEB, +500 mEq kg^{-1} DM. ns, not significant, $p > 0.05$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Values are expressed as the mean per treatment (n = 4) and pooled standard errors of the mean (pSEM).

Supplementary table S5. Progression of digestion of crude protein (CP ADC) in the stomach, proximal, middle, and distal intestine of Atlantic salmon smolt, as affected by contrasting dietary electrolyte balances (dEB) and water salinity.

		FW		SW			*p-values*		
		Low dEB	**High dEB**	**Low dEB**	**High dEB**	**pSEM**	**Salinity**	**Diet**	**Salinity*Diet**
CP ADC (%)	Stomach	16.4	22.6	12.5	23.9	3.39	ns	*	ns
	Proximal	59.9	56.7	65.0	64.2	3.06	ns	ns	ns
	Middle	81.5	79.4	83.3	81.4	1.70	ns	ns	ns
	Distal	82.2	81.0	84.9	86.0	2.42	ns	ns	ns

FW, 0 ppt salinity; SW, 30 ppt salinity; low dEB, -100 mEq kg^{-1} DM; high dEB, +500 mEq kg^{-1} DM. ns, not significant, $p > 0.05$; *, $p < 0.05$. Values are expressed as the mean per treatment (n = 4) and pooled standard errors of the mean (pSEM).

CHAPTER 6

General discussion

This thesis investigates the interplay between dietary and environmental factors on the processes of digestion and osmoregulation in the gastrointestinal tract (GIT) of salmonids. This chapter reflects on the results from previous chapters and explores possible other factors involved in altering these processes. Finally, the implications of this research outcome are given in the context of aquaculture.

6.1 Water fluxes in the gastrointestinal tract of fish postprandial

To better understand the relationship between digestion and osmoregulation in fish postprandial, I first looked at the relative water fluxes in the gastrointestinal tract of freshwater (FW) and seawater (SW) fish, regardless of dietary factors. Figure 6.1A shows a comparison of the relative water fluxes of fish species kept under FW conditions: rainbow trout (*Oncorhynchus mykiss*; Chapters 2 & 3), Atlantic salmon (*Salmo salar*; Chapters 4 & 5), and African catfish (*Clarias gariepinus*; Harter et al., 2013). Different dietary treatments were investigated in the studies reported in Figure 6.1A. The water fluxes in distinct parts of the GIT for each species are averaged across the dietary treatments used in those studies. All three species showed an influx of water in the stomach and proximal intestine, and an efflux in the middle intestine. Depending on the species, in the distal intestine there was either a small efflux or an influx of water. This suggests that the overall pattern of water fluxes in the GIT is similar among these three species under FW conditions, though the magnitude of water fluxes in the segments of the GIT varies among species. Both the influxes and effluxes are larger in smolt Atlantic salmon compared to rainbow trout and African catfish (Figure 6.1A). Figure 6.1B depicts the net water flux through the entire GIT, which is the sum of the relative water fluxes in each of the GIT segments. This net water flux reflects the amount water that is lost via faeces excretion. The net water flux for rainbow trout and African catfish is identical, but for Atlantic salmon it is more than five times higher (Figure 6.1B). This finding

emphasizes that the water dynamics in the GIT of salmon differ from those of trout and African catfish. Further research is needed to determine whether the water dynamics for trout and African catfish are representative of other freshwater fish species.

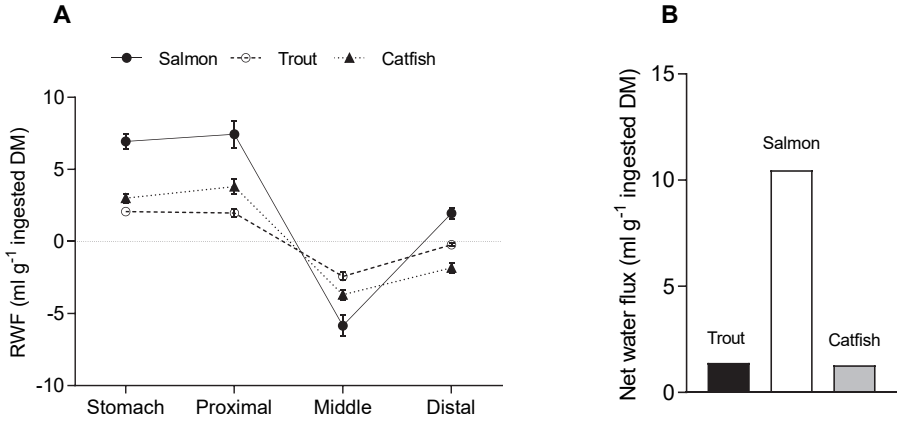

Figure 6.1 Panel A: relative water fluxes (RWF) in the stomach, proximal, middle, and distal intestine of Atlantic salmon (●, solid line), rainbow trout (○, dotted line) and African catfish (▲, dotted line) kept in freshwater, averaged over dietary treatments in the used studies. Data on salmon (tanks, n=12; diets, n=3) and trout (tanks, n=60; diets, n=6) were from this thesis; data on African catfish (tanks, n=12; diets, n=2) from Harter et al., (2013). Panel B: total net water flux: being the summation of the relative water fluxes (both influx and efflux) across the whole gastrointestinal tract (GIT) of fish. Values are expressed as the mean per treatment and standard error (SE).

The differences in water dynamics along the GIT of freshwater fish could be due to species-specific characteristics. For instance, Atlantic salmon may require higher water influx into the stomach and proximal intestine to process ingested food, compared to trout or catfish. Another factor that could alter water fluxes in the GIT in fish body weight (or fish size). The fish species used for comparison in Figure 6.1 had different body weights at the moment of chyme sampling: ~550 g for rainbow trout (Chapter 2 & 3); ~350 g for Atlantic salmon (Chapter 4 & 5), and ~950 g for African catfish (Harter et al., 2013). As a result, the larger water fluxes in the GIT of salmon may be related

to the lower fish weight. The higher influx of water into the stomach of Atlantic salmon might suggest a higher water drinking rate or endogenous secretions. However, in the abovementioned studies, no distinction was made between endogenous or exogenous water fluxes in the stomach (drinking versus secretions). Bucking and Wood (2006) suggested that some water ingestion occurs during feeding in rainbow trout (kept in freshwater), but it is mainly accidental. Kristiansen and Rankin (2001) proposed that, drinking may occur during feeding in FW environment, although it is only linked to food moisturizing rather than any osmoregulatory requirement for water. The findings of the present study compared with literature suggest that smaller fish show larger water influx in the stomach and proximal intestine. This could be related to facilitating food processing and digestion in the GIT. However, more research is needed to determine whether there is an underlying mechanism linking fish weight to water fluxes.

It could be argued that the difference in water fluxes between Atlantic salmon, rainbow trout and African catfish (Figure 6.1B) is related to the use of smolts salmon as experimental animal in this thesis (Chapter 4 & 5). Smolts may be experiencing pre-adaptive changes in intestinal fluid transport associated with the development of the migratory phase (smoltification) to seawater (SW). A long-term study by Usher et al. (1991), which measured water fluxes in FW Atlantic salmon before and after smoltification, found that FW late smolts had larger amounts of fluid transport in the intestine than parr and early smolts. Usher et al. (1991) proposed that the gradual increase in intestinal fluid transport during smolting in FW Atlantic salmon is independent from drinking, which occurs after transfer to SW. Accordingly, the higher magnitude of water fluxes observed in Atlantic salmon (Figure 6.1) may be due to the use of smolts, which increased endogenous water secretions and absorption to prepare for the life stage in seawater.

Factors related to feeding (i.e., diet type, pellet size and feeding level) may also have contributed to the differences in water dynamics shown in Figure 6.1. In the studies presented in Figure 6.1, rainbow trout and African catfish

were fed restrictively, whereas Atlantic salmon were fed to satiation. Therefore, feeding level may also be the reason for the observed differences among species in the magnitude of water dynamics. Furthermore, differences in dietary composition across studies could have further altered water fluxes in the GIT postprandial. Indeed, the findings of the present thesis (Chapter 2, 3 & 5) and literature (Elesho et al., 2022; Harter et al., 2013), show that diet composition influences water fluxes in the stomach and proximal intestine of FW fish (Figure 6.2). The influence of dietary factors on water fluxes in the GIT of freshwater fish is discussed later in this chapter.

In seawater condition, fish ingest water to maintain a constant internal osmotic value. Literature on water fluxes in the GIT of seawater teleosts is extremely limited (Bucking et al., 2011; Kirsch, 1978; Parmelee and Renfro, 1983; Potts et al., 1970; Usher et al., 1991). However, there are few more studies that measured drinking rate in seawater-adapted fish. Perrott et al. (1992) measured drinking rates in different fish species (non-fed) under freshwater and seawater condition. They showed that water ingestion in full-strength seawater fish was 10-100 times higher than in freshwater fish. Even though all seawater fish drank large volumes of water, the rates varied considerably between species. Water drinking rates reported in literature for teleosts living in freshwater or seawater environments are summarized in Table 6.1.

Table 6.1. Water drinking rates (ml kg^{-1} h^{-1}) in unfed freshwater and unfed seawater-adapted fish.

Fresh water	**Drinking rate (ml kg^{-1} h^{-1})**
Carp (*Cyprinus carpio*)[1]	0.03
Catfish (*Clarias gariepinus*)[1]	0.21
Eel (*Anguilla anguilla*)[1]	0.09
Flounder (*Platichthys flesus*)[1]	0.04
Rainbow trout (*Oncorhynchus mykiss*)[1]	0.07
Atlantic salmon (*Salmo salar*)[10,11,12]	0.58
Seawater	
Rainbow trout (*Oncorhynchus mykiss*)[1,2,3]	1.77
Atlantic salmon (*Salmo salar*)[3,4,5,6,7,8]	3.12
Brown trout (*Salmo trutta*)[9]	1.20
Eel (*Anguilla anguilla*)[1]	1.00
European flounder (*Platichthys flesus*)[1]	1.15
Plaice (*Pleuronectes platessa*)[1]	2.52
Dab (*Limanda limanda*)[1]	3.60
Whiting (*Merlangius merlangus*)[1]	1.80
Sea scorpion (*Myxocephalus scorpius*)[1]	7.76
Pogge (*Agonus cataphractus*)[1]	2.21
Wolf fish (*Anarhicus lupis*)[1]	2.24
Sand eel (*Ammodytes lanceolatus*)[1]	2.96

The estimated drinking rates were measured by: [1]Perrot et al. (1992); [2]Wilson et al. (1996); [3]Bucking et al. (2011); [4]Usher et al. (1988); [5]Smith et al. (1991); [6]Lega et al. (1992); [7]Fuentes et al. (1996a); [8]Potts et al. (1970); [9]Nielsen et al. (1999); [10]Eddy (2007); [11]Fuentes et al. (1996b); [12]Fuentes and Eddy (1997).

Non-fed seawater fish ingest more water than freshwater fish, corroborating previous research on the importance of water ingestion in SW fish for osmoregulation. However, the large variability in drinking rate may indicate that, in seawater conditions, factors that are not necessarily related to feeding may affect osmoregulation and thus water ingestion. Evolutionary theories posit that the GIT played a pivotal role when teleost fish re-entered seawater approximately 150-200 million years ago after evolving in freshwater (Fyhn et al., 1999). Early teleosts engaged in sporadic forays into seawater for feeding while returning to freshwater for spawning (diadromy). This behavior, as exemplified by salmonids, enabled embryos to inherit adaptations for freshwater life, avoiding immediate challenges of osmoregulation in seawater (Fyhn et al., 1999). The key to diadromy likely lies in genes associated with migration signaling, linked to nutrient

assimilation, suggesting that the digestive tract serves as a focal point for adaptations to shifting environmental salinity (Delgado and Ruzzante, 2020). It is now recognized that, depending on water salinity, the GIT plays a role in both digestion and osmoregulation. The GIT is not involved in osmoregulation in FW conditions. As a result, I hypothesize that changes in water fluxes in the GIT of FW fish are related to feeding and digestion. A small amount of water is ingested during feeding. In conclusion, the findings of this thesis and previous research indicate that water influx in the GIT of freshwater fish is primarily caused by endogenous secretions rather than water ingestion, and that fish limit the amount of water that enters the GIT.

6.2 Dietary effect on water fluxes: freshwater fish

In this thesis, the influence of dietary factors on osmoregulatory processes within the GIT of freshwater rainbow trout (Chapters 2 & 3) and Atlantic salmon (Chapters 4 & 5) was investigated. Figure 6.2 summarizes the water fluxes measured in the GIT of freshwater fish tested in this thesis. Dietary factors influenced water fluxes differently: electrolyte balance (dEB) and protein-to-energy ratio (P:E) altered water fluxes in rainbow trout, whereas dietary buffering capacity ($CaCO_3$) and dEB had no effect on water fluxes in rainbow trout and Atlantic salmon, respectively. Furthermore, in all experiments, the diet had a significant effect on water fluxes only in the stomach and, sometimes, the proximal intestine, but not in the middle or distal intestine.

Interestingly, the studies presented in Figure 6.2 show a consistent pattern of higher or lower water influx into the stomach (depending on the dietary factor), which is counterbalanced by a higher or lower water influx into the proximal intestine. This mechanism is evidenced by the intersecting lines between stomach and proximal intestine for all dietary treatments presented in panels A-E (Figure 6.2) and suggests the presence of an interplay between these two segments concerning water fluxes.

This hypothesis is in line with the observations of Harter et al. (2013), who also reported a negative correlation between water influxes in the stomach and proximal intestine in freshwater African catfish. To further visualize this relationship, the values of water influx in the stomach versus proximal intestine in trout and salmon (this thesis) were plotted and compared to those in African catfish by Harter et al. (2013) (Figure 6.3). Figure 6.3 shows that this reciprocal relationship is consistent across the three species. This is also reflected by the fact that the slopes of the lines for all three species (Atlantic salmon, African catfish, and rainbow trout) are similar (-1.26, -1.29, -1.19, respectively; $p > 0.5$), suggesting a general trend. Averaged across these fish species, the water influx in the proximal intestine decreases by 1.2 unit when the water influx in the stomach increases by 1 unit (ml g^{-1} ingested DM) ($p < 0.05$).

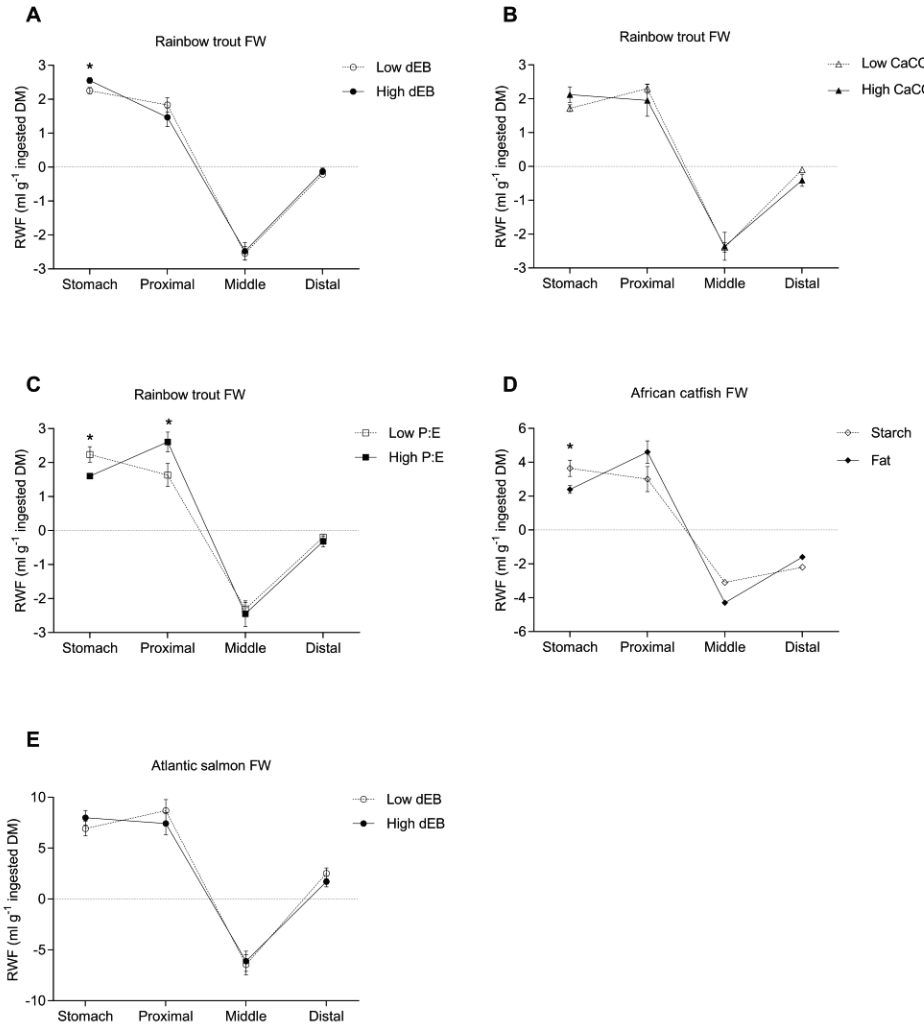

Figure 6.2. Relative water fluxes (RWF) in the stomach, proximal, middle and distal intestine of rainbow trout (Chapter 2, 3; panels A, n=6; B, n=12; C, n=12), African catfish (Harter et al., 2013; panel D, n=6), smolt Atlantic salmon (Chapter 5, panel E, n=4) kept in freshwater (FW) as affected by diets having contrasting dietary factors. dEB, dietary electrolyte balance; $CaCO_3$, calcium carbonate; P:E, protein-to-energy ratio; starch versus fat diet. The asterisk (*) indicate the presence of a significant effect of dietary treatment ($p < 0.05$). Values are expressed as the mean per treatment and standard error (SE).

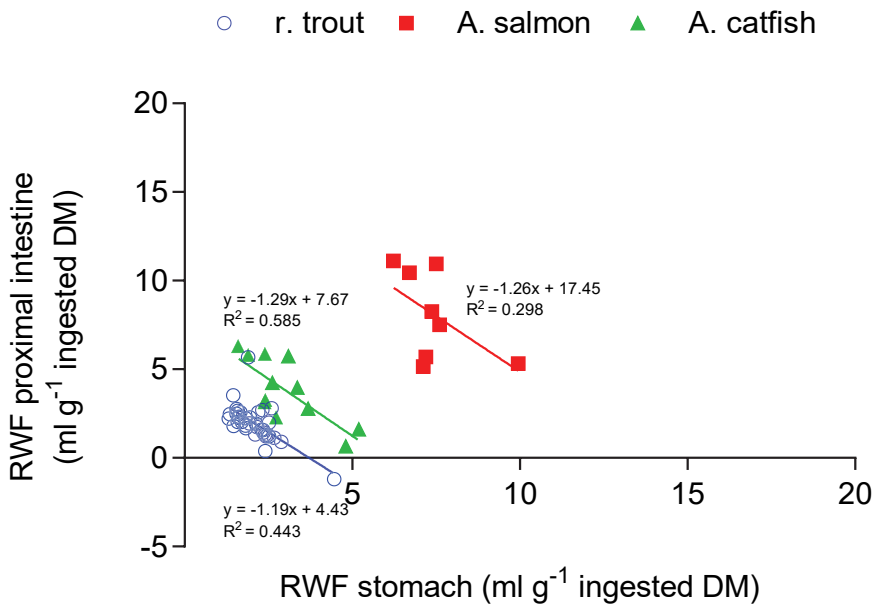

Figure 6.3. Postprandial relative water flux (RWF) in proximal intestine and stomach in fish kept in freshwater (FW). Data on rainbow trout (Chapter 2 & 3; n=60), Atlantic salmon (Chapter 5; n=8) and African catfish (Harter et al., 2013; n=12).

Furthermore, the net water flux (sum of the relative water fluxes across the GIT) calculated in freshwater trout and salmon (this thesis, Chapter 2, 3 & 5) and in catfish (Harter et al., 2013) are shown in Figure 6.3. The net water flux was not affected by dietary treatments ($p > 0.05$), except for trout fed diets with contrasting level of $CaCO_3$ (Figure 6.4). Despite being significant, the impact of dietary buffering capacity was in absolute terms small on the net water flux. Overall, these observations suggest that in freshwater conditions, the effect of dietary factors on the net water flux (amount of water leaving the gut via faeces) is either absent or marginal.

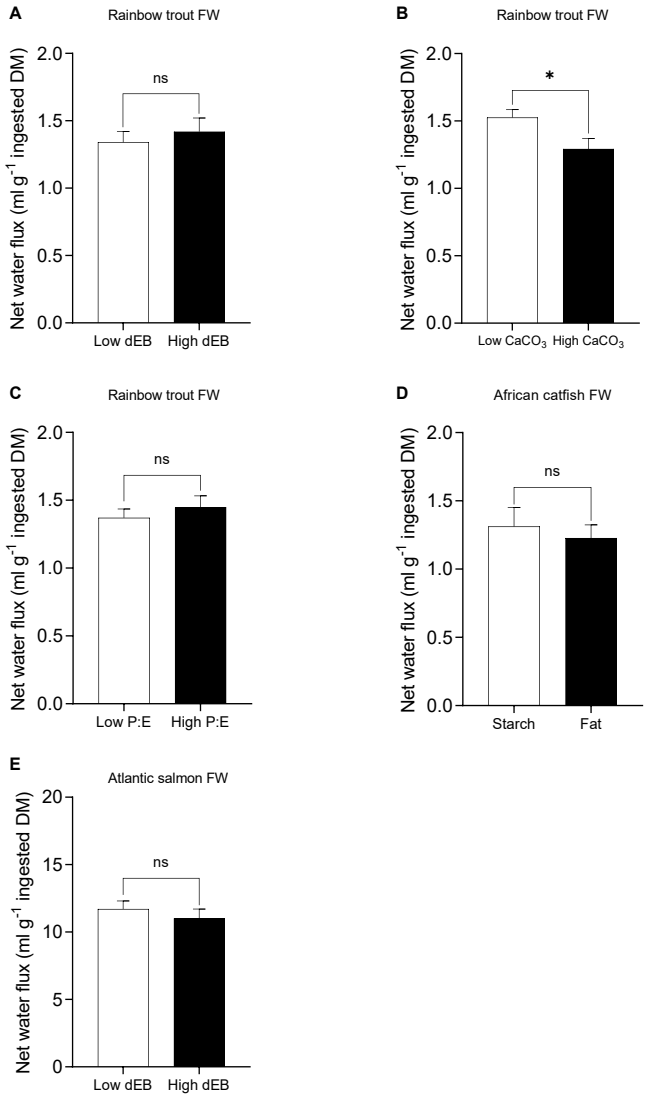

Figure 6.4. Total net water flux: being the summation of the relative water fluxes (both influx and efflux) across the whole gastrointestinal tract (GIT) of fish kept in freshwater (FW): rainbow trout (Chapter 2, 3; panels A, n=6; B, n=12; C, n=12), African catfish (Harter et al., 2013; panel D, n=6) and Atlantic salmon (Chapter 5, panel E, n=4); as affected by diets having contrasting dietary factors: dEB, dietary electrolyte balance; CaCO3 content, calcium carbonate; P:E, dietary protein-to-energy ratio; starch versus fat diet. The asterisk (*) indicate the presence of a significant effect ($p < 0.05$) of dietary treatment. Values are expressed as the mean per treatment and standard error (SE).

These findings collectively demonstrate that the interplay between the stomach and proximal intestine plays a role in regulating water movement in the GIT of freshwater fish. This further supports the hypothesis that water ingestion in freshwater fish is not a deliberate action aimed at moisturizing food in the stomach. Instead, it appears to occur accidentally during feeding, and the presence of water influx in the stomach triggers a compensatory mechanism in the proximal intestine (to limit water ingestion). This conclusion strengthens the hypothesis that fish in freshwater strive to minimize water uptake from their environment, as they must subsequently expend energy to excrete this excess water (as diluted urine) to maintain osmoregulation.

6.3 Dietary effect on water fluxes: seawater fish

In this paragraph, the effect of dietary factors on water fluxes along the GIT of seawater-adapted fish is discussed. In Atlantic salmon fed diets contrasting in dEB (Chapter 5), water fluxes in the GIT were significantly different between treatments (Figure 6.5C). In contrast to freshwater-adapted salmon, the diet effect on water influx in the stomach was not counterbalanced in the proximal intestine (as discussed in section 6.2). In seawater-adapted salmon, water influx into the stomach was followed by more water influx into the proximal intestine when fed the low dEB diet (Chapter 5; Figure 6.5C). In Chapter 5, relative drinking rate increased from 2.05 to 3.07 ml kg^{-1} h^{-1} g^{-1} ingested DM when fish were fed the high and the low dEB diet, respectively. Furthermore, dEB affected water uptake in the middle intestine of seawater-adapted salmon. These changes in water fluxes along the GIT, as well as drinking rate, could be attributed to a homeostatic imbalance caused by diet. Philip et al. (2022) measured carbonate precipitation ($CaCO_3$ and $MgCO_3$) in the gut of Atlantic salmon fed low and high levels of dEB after 24 hours in seawater. The presence of these precipitates was used as an indirect indicator of water ingestion. According to Philip et al. (2022), salmon fed the low dEB diet had more precipitate formation in the gut. As a result, the authors

hypothesized that fish fed the low dEB diet drank more seawater. While literature is lacking in supporting studies, the findings of Philip et al. (2022) provide additional support for our observation that a low dEB diet leads to increased drinking in seawater-adapted Atlantic salmon. Additionally, net water flux in SW-adapted salmon fed contrasting dEB was calculated in the present thesis (Figure 6.5D). Data show that net water flux was significantly higher in fish fed the low dEB diet. This implies that dietary factors can influence the amount of water leaving the GIT via faeces in seawater-adapted fish, which is in contrast to the findings in freshwater fish (Figure 6.4, 6.5B). The observed differences in water drinking rate and water fluxes in the GIT suggest that fish regulate water intake in response to changes in osmoregulation caused by the diet. Suboptimal dEB levels may drive to higher or lower drinking rate. Notably, drinking rate in unfed seawater-adapted fish is about 2 ml kg^{-1} h^{-1}, but it may increase when fish are exposed to stress conditions (Marshall and Grosell, 2005). Previous research has shown that stress can alter gill permeability to water and ions, leading to increased water loss via the gills in seawater-adapted fish (Mazeaud et al., 1977; Portz et al., 2006; Wendelaar Bonga, 1997). An unbalanced diet may induce stress which, in turn, can disrupt fish metabolism and increase oxygen demand (Saravanan et al., 2013a). However, also a balanced diet, that increases feed intake, can lead to higher metabolism, and subsequently increase oxygen demand and consumption. In both cases, the elevated oxygen demand likely drives to increased water loss through the gills due to respiration in seawater. Consequently, both unbalanced and optimal diets may cause higher drinking rates to compensate for the increased water loss from the gills. As a result, a higher drinking rate in fish may indicate physiological stress caused by an unbalanced diet, but it may also indicate optimal performance in fish fed an efficient diet.

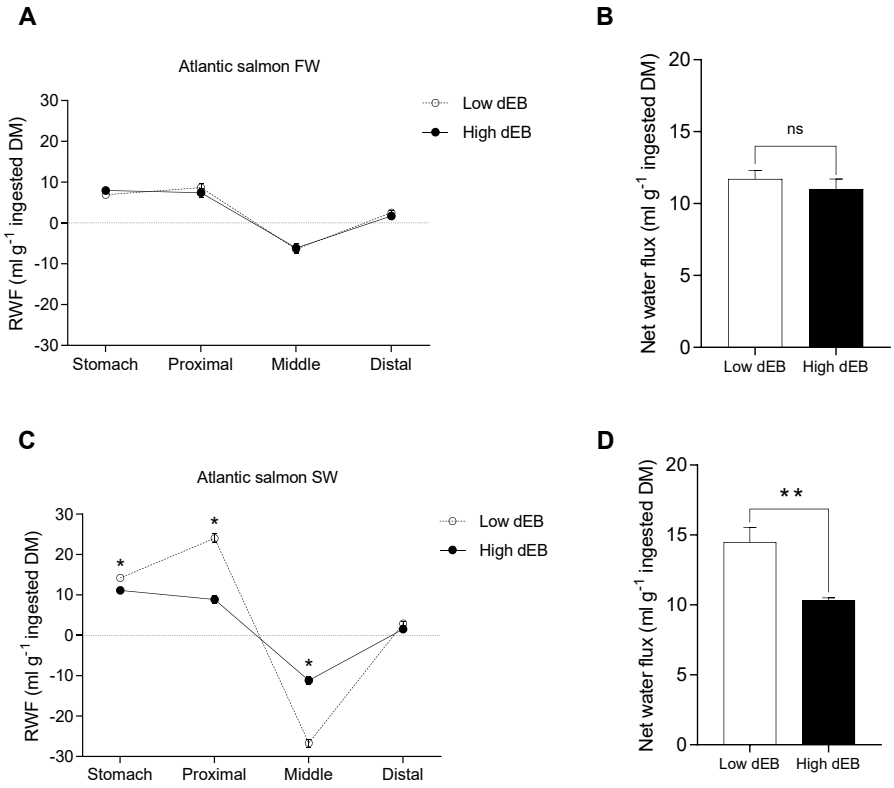

Figure 6.5. Relative water fluxes (RWF) and net water flux measured in freshwater- (FW) and seawater- (SW) adapted smolt Atlantic salmon (Chapter 5). In the left panels, the empty dots (○) and dotted line represent the low dEB diet and the full dots (●) and continuous line represent the high dEB diets. The asterisk (*) indicates the presence of a significant effect of dietary treatment. In the right panels, the net water flux as affected by dietary treatments is depicted. ns, not significant, $p > 0.05$; **, $p < 0.01$. Values are expressed as the mean per treatment ($n = 4$) and standard error (SE).

To summarize, in freshwater conditions, dietary factors primarily influence water fluxes in the stomach and proximal intestine, and these effects frequently exhibit an opposite interplay. For instance, increased water influx in the stomach is usually followed by decreased water influx in the proximal intestine. In seawater conditions, dietary factors have a larger impact on drinking rate, resulting in differences in water absorption in the middle intestine and in net water flux throughout the GIT.

6.4 Drinking rate: water salinity and dietary effects

As mentioned above, drinking rate in fish highly differ between freshwater and seawater environment. In freshwater, drinking rate is minimal and primarily associated with feeding, whereas in seawater, fish actively drink for osmoregulation (Whittamore, 2012; Zydlewski and Wilkie, 2012). Results from this thesis (Chapter 4 & 5) and literature show that drinking rate increases with water salinity (Table 6.1; Grosell, 2010). However, very few studies have addressed a dose response relationship between water salinity and drinking rate. To assess this relationship, data from literature (Eddy, 2007; Fuentes et al., 1996a; Fuentes and Eddy, 1997; J. Fuentes et al., 1999; Kristiansen and Rankin, 2001; Lega et al., 1992; Nielsen et al., 1999; Perrott et al., 1992; Philip et al., 2022; Pyle et al., 2003; Ruohonen et al., 1997; Smith et al., 1991; Usher et al., 1988; Wilson et al., 1996; Wood and Bucking, 2010) on water drinking rate in salmonids fish (salmon and trout) under different water salinities were combined with data from this thesis on Atlantic salmon smolt (Chapter 4 & 5) and depicted in Figure 6.6. Fish drinking rate in freshwater condition ranges between 0 and 2 ml kg^{-1} h^{-1}, regardless of whether fish are fed or not, but it is slightly higher when fish are fed (Figure 6.6). In seawater condition, drinking rate showed a larger variability, ranging between 1.4 and 7.9 ml kg^{-1} h^{-1} above 30 ppt salinity. However, statistical analysis of the dataset depicted in Figure 6.6 show that the relationship between salinity and drinking rate did not differ ($p > 0.05$) between fed and non-fed salmonids. Moreover, no significant differences ($p > 0.5$) in drinking rate were present between fish species (salmon versus. trout). In all studies (Figure 6.6), drinking rate increased linearly with salinity ($p < 0.001$), with an R^2 of 0.34. This implies that water salinity accounts for only 34% of the variation in drinking rate, suggesting that other factors influence water drinking rate (e.g., fish body weight, dietary and/or environmental factors). Diets with different dEB caused differences in drinking rate in salmon kept in SW (Chapter 5). Further investigation is required to determine whether

other differences in dietary composition can influence the drinking rate under seawater conditions.

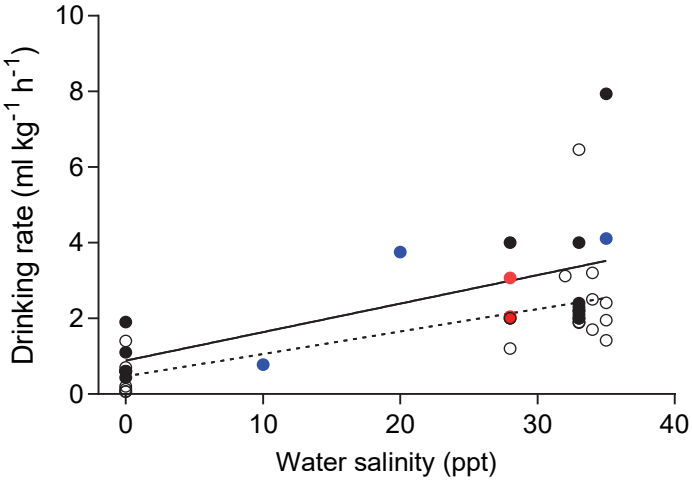

Figure 6.6. Water drinking rates (ml kg^{-1} h^{-1}) in salmonids (Atlantic salmon and rainbow trout) as affected by increasing water salinity (ppt). Black and white dots represent data from literature where: ○ = unfed, ● = fed. The coloured dots represent data from the present study (blue dots, Chapter 4; red dots, Chapter 5). Full trend line refers to fed fish; dotted trend line refers to unfed fish. Constructed dataset by literature (see main text) and from this thesis. Equation, intercept (SE) ± β (SE): unfed, Y = 0.059 (0.014) X + 0.468 (0.367) (R^2 = 0.47); fed, Y = 0.075 (0.025) X + 0.885 (0.672) (R^2 = 0.36).

Fuentes and Eddy (1997) showed that drinking rate (ml kg^{-1} h^{-1}) declined with fish size in juvenile Atlantic salmon adapted to freshwater. The dataset presented in Figure 6.6 was also used to determine whether water drinking rate across studies is related to fish size (Figure 6.7). Figure 6.7 shows that drinking rate (ml kg^{-1} h^{-1}) do not correlate ($p > 0.5$) with body weight across salmonid studies. Feeding regimens and water salinities, however, vary between these studies. As a result, to better investigate the interplay between drinking rate and fish body size further research is needed.

Water temperature can also influence drinking rates in teleosts. Previous research has shown that drinking rate in fish decreases with low temperatures: for example, in European eel (*Anguilla anguilla*), drinking rate decreased from 29.9 ml kg^{-1} day^{-1} at 15 °C to 6.5 ml kg^{-1} day^{-1} at 5 °C (Motais and Isaia, 1972); in flounders (*Platichthys flesus*), drinking rate decreased from 46.0 ml kg^{-1} day^{-1} at 16 °C to 15.6 ml kg^{-1} day^{-1} at 6 °C (Maetz and Evans, 1972). In ectotherm organisms, the metabolic rate is determined by the ambient temperature within the temperature tolerance range. The higher the temperature, the faster the metabolic rate and the higher the drinking rate. As a result, high water temperatures increase oxygen demand (Motais and Isaia, 1972). Nonetheless, low water temperatures reduce fish metabolism and thus oxygen consumption. Furthermore, if the water temperature falls below the optimum range and becomes too cold, fish may experience stress. During the winter, salmon kept in sea cages may be exposed to temperatures below their preferred natural habitat range of 4 to 12 °C (Saunders, 1986). Lega et al. (1992) found that, as seawater temperature decreased (from 5.6 to 1 °C), drinking rate in unfed Atlantic salmon dropped (from 13.9 to 5.7 ml kg^{-1} day^{-1}), and suggested that water temperature can be a major stressor for fish, leading to osmotic imbalances in the gastrointestinal tract. These findings indicate that water temperature can affect fish osmoregulation by influencing water fluxes. As a result, changes in water temperature may cause changes in drinking rate, raising the question of when (and in what context) increased or decreased drinking rate indicates fish fitness or stress. Overall, a variety of factors can influence drinking rate and water fluxes in fish GIT, emphasizing the complexities of these processes.

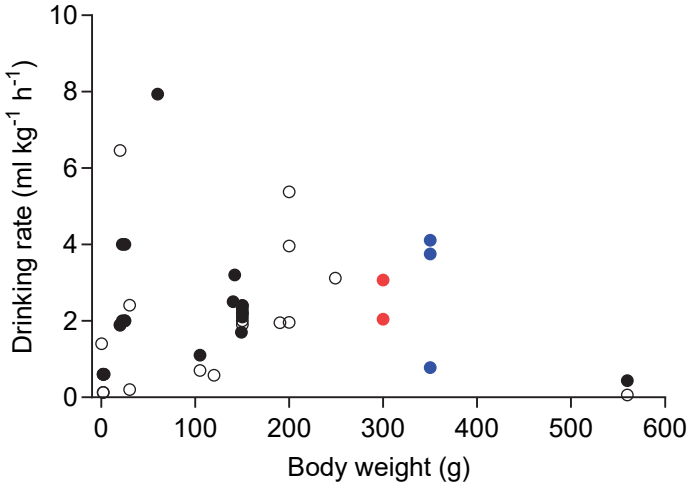

Figure 6.7. Drinking rates (ml kg^{-1} h^{-1}) in salmonids as affected by body weight (g). Black and white dots represent data from literature where: ○ = unfed, ● = fed. The coloured dots represent data from the present study (Blue dots, chapter 4; red dots, Chapter 5). Constructed dataset by literature (see main text) and from this thesis.

In Chapter 4, as drinking rate increased with water salinity, water influx in the stomach of smolt Atlantic salmon remained similar across salinities. In contrast, water influx in the proximal intestine increased by more than fourfold between 0 and 35 ppt, from 6.2 to 27.3 ml g^{-1} ingested DM. This led to the hypothesis that, at high salinities, most of the ingested water bypasses the stomach and moves quickly to the proximal intestine. In the proximal intestine, endogenous secretions combine with ingested seawater, contributing to further increase water influx (Grosell, 2010). In a study on tilapia (*Alcolapia grahami*) living in the extreme environmental conditions of Lake Magadi (high carbonate alkalinity = 245 mEq l^{-1} and pH = 9.85), it was found that ingested water bypasses the stomach during both starvation and feeding conditions and moves directly to the proximal intestine (Bergman et al., 2003). Based on these findings, I hypothesize that in seawater conditions, ingested water serves primarily for osmoregulation in the intestinal segments rather than moisturizing the food in the stomach.

6.5 Digestion kinetic: water salinity and dietary effect

Figure 6.8 summarizes the results on the kinetics of crude protein digestion (CP ADC) obtained in this thesis as affected by dietary factors for salmon and trout, and Figure 6.9 summarizes the effects of water salinity on CP ADC in salmon. These figures show the main effects. Generalizing to freshwater conditions, dietary factors affected the kinetics of CP ADC, but the absolute differences in CP ADC were minimal. Furthermore, differences in water fluxes between dietary treatments in FW (Figure 6.2) did not correlate with differences in CP ADC kinetics. For example, dietary $CaCO_3$ supplementation influenced CP ADC kinetics (Figure 6.8B) but had no effect on water fluxes (Figure 6.2B). The dietary P:E ratio, on the other hand, had a clear effect on water fluxes in FW rainbow trout (Figure 6.2C), but no effect on digestion kinetics (Figure 6.8C). Similarly, dEB had an impact on CP ADC in the middle intestine of rainbow trout (Figure 6.8A), but no effect on water fluxes (Figure 6.2A). The findings of this thesis indicate that differences in water fluxes in the GIT due to diet composition do not translate into differences in CP digestion kinetics in freshwater. Furthermore, when differences in faecal digestibility were found between dietary treatments in rainbow trout in FW (dEB and P:E: Chapters 2 & 3), no differences in net water fluxes were observed.

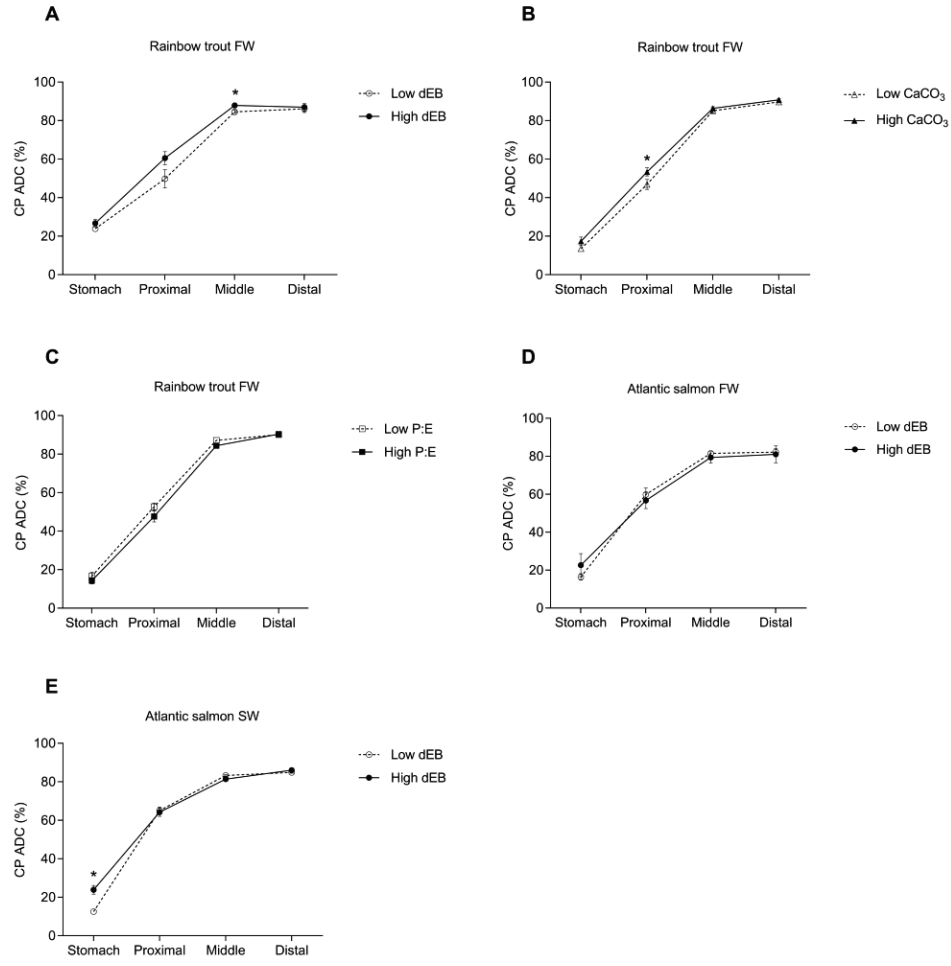

Figure 6.8. Kinetic of crude protein digestion (CP ADC %) in the stomach, proximal, middle and distal intestine of freshwater (FW) rainbow trout (Chapter 2, 3; panels A, n=6; B, n=12; C, n=12), smolt Atlantic salmon (Chapter 5, panel D, n=4), and seawater (SW) smolt Atlantic salmon (Chapter 5, panel E, n=4), as affected by contrasting dietary factors. dEB, dietary electrolyte balance; CaCO3, calcium carbonate; P:E, protein to energy ratio. The asterisk (*) indicates the presence of a significant effect of dietary treatment. Values are expressed as the mean per treatment and standard error (SE).

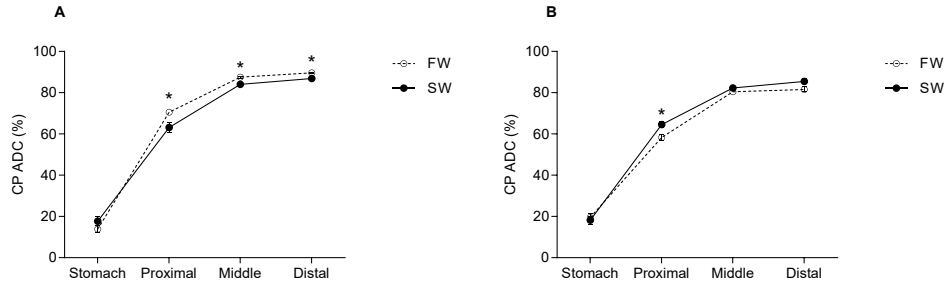

Figure 6.9. Kinetic of crude protein digestion (CP ADC %) in the stomach, proximal, middle, and distal intestine as affected by water salinity. Left panel, Chapter 4, n=4; Right panel, Chapter 5, n=8. The empty dots (○) and dotted line represent freshwater (FW) condition and the full dots (●) and continuous line represent seawater (SW) condition. The asterisk (*) indicates the presence of a significant effect of dietary treatment. Values are expressed as the mean per treatment and standard error (SE).

The effect of salinity on CP ADC kinetics in salmon was studied in Chapters 4 and 5, and the results are summarized in Figure 6.9. The influence of salinity varied between the two studies. In Chapter 4, CP digestibility decreased as water salinity increased in all intestinal segments of salmon fed a commercial diet (Figure 6.8A). In contrast, In Chapter 5, salmon fed contrasting dEB diets showed higher CP ADC in all intestinal segments in seawater compared to freshwater conditions (Figure 6.8B). Salinity has an inconsistent effect on the kinetics of CP digestion in the gastrointestinal tract, even though it clearly increases water fluxes, implying that water fluxes and CP digestion kinetics are unrelated in SW. Furthermore, dEB affected CP ADC in the stomach (i.e., the faster movement of solubilized proteins from the stomach to the proximal intestine) of salmon in SW, which was higher in fish fed a high dEB diet than a low dEB diet (Figure 6.8E). However, no dietary effect was observed in the intestine. In contrast, dEB influenced water fluxes in the stomach and intestine of SW-adapted fish (Figure 6.4C). This suggests that the diet may influence drinking rate in seawater fish but has little effect on digestion kinetics in the intestine. Furthermore, in both Chapter 4 and 5, it was proposed that ingested seawater in salmon smolts bypasses the stomach and enters the

proximal intestine. Overall, the findings of this thesis show that dietary factors have little or no effect on water fluxes and digestion kinetics in freshwater, and that these processes are independent of one another. Similarly, in seawater conditions, dietary factors have no effect on kinetic digestion, except the stomach, where differences can be attributed to the diet effect on chyme pH rather than water influx. Furthermore, the effect of water salinity on CP ADC kinetics is variable, making it difficult to identify the underlying causes. Overall, these findings show that the effects of diet and environment on water fluxes and digestion kinetics are not correlated and may be completely independent processes. This hypothesis is further elaborated in the following paragraph.

6.6 Digestion and osmoregulation in seawater fish GIT

Based on the findings of the current thesis (Chapters 4 & 5), it is hypothesized that in seawater-adapted fish, the GIT has evolved the ability to alternate digestion and osmoregulation processes to optimize them. There is some evidence in the literature to support this hypothesis. A recent study on naked carp (*Gymnocypris przewalskii*) living in the highly alkaline and saline Lake Qinghai (carbonate alkalinity, 29 mmol l^{-1}; pH = 9.3; salinity 13-15 ppt) revealed that digestive and drinking behaviors are distinct events, and the intestine alternates between these functions with a distinct daily rhythm (Wang et al., 2022). They observed that drinking rate is higher at night, while feeding mainly occurs during the day. The daily rhythm appears to be an adaptive strategy for naked carp in the challenging environmental conditions of Lake Qinghai to deal with the conflicting demands of feeding/digestion and drinking/osmoregulation. This suggests that osmoregulation and digestion in fish gastrointestinal tracts can be influenced by daily rhythms and are sensitive to different environmental factors. Yúfera et al. (2014) observed a clear daily pattern in gilthead seabream (*Sparus aurata*) when fed a single daily meal, with the stomach filling immediately after the initial meal and emptying during the night, resembling a more natural feeding regime.

Additionally, Yúfera et al. (2014) measured changes in stomach fullness when fish were continuously fed for 12 hours during the light period, similar to intensive farming conditions. The study found two peaks of food intake (morning and afternoon) with a minimum level of feed intake during the day and almost none during the night, indicating that even with continuous feeding, fish tend to concentrate their feed intake at specific times. This feeding behavior may be related to the time required for stomach emptying and the physiological need to regulate ion and water balances in the gastrointestinal tract. Furthermore, previous research suggested that changes in pH in the intestine of gilthead seabream are influenced more by the light/dark pattern than the feeding protocol (Montoya et al., 2010; Yúfera et al., 2014). Montoya et al. (2010) observed a peak in alkaline protease activity in the intestine six hours after the light turned on (at 8:00), even though the meal was provided seven hours later. As a result, the regulation of intestinal digestion seems independent of the feeding protocol. This indicates that the intestine may be able to modulate the digestive and osmoregulation processes to establish a 24-hour cycle.

Individual variations in the amount of water/dry matter within the different segments of the GIT were frequently observed visually at the moment of chyme sampling during all experiments in this thesis, but this was particularly evident in SW fish. In the future, it would be helpful to sample fish at different times of day and night to determine whether there are any significant differences in digestion parameters (e.g., chyme pH, enzyme activity) and osmoregulation (water fluxes) between sampling times.

6.7 Implications for farmed fish

In commercial aquaculture, fish are fed pelleted diets at high feeding levels, which strongly differs from their natural food item. Carnivorous fish, like salmon and trout experience intermittent feeding depending on prey capture success, while in aquaculture practices feed is often continuously supplied. Furthermore, commercial pelleted fish feeds contain about 90% DM, while

many natural preys contain 20-30% DM (Jobling, 1986; Kristiansen and Rankin, 2001). This raises concerns about how dietary factors affect gastrointestinal tract digestibility and osmoregulatory functions. In farming conditions, pellets must be moistened after ingestion due to their high DM content. Endogenous water influxes may make it difficult to moisturize the pellet in FW freshwater-adapted fish, which avoid drinking water as they live in a hypo-osmotic environment. Dietary factors (e.g., dry matter, electrolyte balance, buffering capacity, etc.) may further challenge or aid food moisturization in FW conditions. However, the current study (Chapters 2 & 3) found that dietary factors had no influence on water dynamics in the GIT of freshwater fish. Furthermore, the osmoregulatory function of the GIT in FW condition appears to be only related to ion uptake from the diet. Previous research has shown that supplementing diets with extra minerals might aid fish adaptation to seawater conditions and improve survival upon transfer from FW to SW (smoltification) (Basulto, 1976; Pellertier and Besner, 1992; Philip et al., 2022; Zaugg, 1982; Zaugg et al., 1983). In the present thesis (Chapter 5), dEB did not impact drinking rate in freshwater-adapted Atlantic salmon, but significantly affected drinking rate in SW conditions. While it is still worthwhile to investigate other dietary factors that can influence water fluxes in the GIT of freshwater-adapted fish, the results of this thesis show that the effect of dietary factors in FW conditions cannot be directly translated to SW conditions. Dietary factors can have different effects on water dynamics in the GIT depending on the salinity. Diets with different dEB in seawater alter drinking rate (Chapter 5), which has a direct impact on osmoregulation. This raises the question of whether inducing higher or lower drinking rates in seawater-adapted fish through changes to diet formulation is beneficial or disadvantageous.

According to existing studies, starved fish consume less water compared to fed fish (Eddy, 2007; Pyle et al., 2003; Usher et al., 1988). Starved fish have a slower metabolic rate, which leads to a lower drinking rate. As a result, food consumption promotes drinking rate in fish. Further, dietary characteristics

can alter the magnitude of drinking independently from the amount of ingested feed. Indeed, in Chapter 5, salmon fed a high dEB diet drank less water despite consuming more food. This observation highlights the influence of dietary factors beyond feeding on water ingestion, indicating that the relationship between feeding and drinking is not always linear.

Previous research demonstrates that feed intake in fish is influenced by both dietary and environmental factors (Kestemont and Baras, 2001; Saravanan et al., 2012). A diet that stimulates higher feed intake also increases fish metabolism and oxygen consumption (Saravanan et al., 2012). This metabolic response leads to increased water loss through the gills, as proposed by Marshall and Grosell (2005). As a compensatory mechanism, fish may increase their drinking rate to maintain homeostasis. However, as mentioned above, in smolt Atlantic salmon fed the low dEB diet (Chapter 5) feed intake was lower, despite a higher drinking rate. This suggests that the diet may cause metabolic stress in the fish, altering their metabolism (maintenance requirement for energy), and require higher oxygen consumption to cope with the stress. This larger oxygen demand could, in turn, lead to increased drinking rate.

As a result, I propose that drinking rate in seawater fish is involved in maintaining osmoregulation rather than aiding digestion, and that it is related to oxygen metabolism (respiration). Therefore, from an aquaculture perspective, it may be useful to investigate other factors (dietary, environmental) that may alter fish osmoregulation and metabolism.

Among environmental factors, changes in water dissolved oxygen and temperature may be major stressors affecting fish metabolism during prandial activity. High water temperature increases fish metabolic rate, which in turn increases drinking rate. Moreover, high water temperature corresponds to lower dissolved oxygen, which causes stress in the fish, necessitating a further increase in drinking. In contrast, low water temperature slows fish metabolism, resulting in lower drinking rates. Furthermore, when water temperature falls below the optimum level, fish become stressed, which

reduces their drinking rate (Lega et al., 1992). As a result, it may be worthwhile to measure drinking rate, oxygen consumption, and kinetic water and nutrient fluxes in the GIT of seawater fish at low and high temperatures. This could help determine to what extent oxygen deprivation affects osmoregulation (water fluxes) in the GIT of fish and the kinetics of nutrient digestion. To sum up, fish metabolism can increase in response to both higher food intake and stress (e.g., an unbalanced diet or suboptimal temperature). However, more research is needed to determine when increased drinking rate reflects fish fitness or stress.

Based on the hypothesis that osmoregulation (drinking rate) and digestion are two distinct events in the GIT of seawater fish, it would be interesting to conduct a study in which fish are sampled at different times of the day/night and drinking rate and digestion kinetics are measured to see if there is a circadian rhythm in alternating these two processes. Even if these GIT functions are distinct, feeding copious amounts of dry pellets in an intensive farming setting may impair fish's ability to alternate between non-drinking (digestion) and drinking events, and their efficiency. As a result, fish metabolism may be negatively impacted and lead to poor growth performance. Thus, developing effective feeding strategies in aquaculture requires a thorough understanding of how feeding practices and dietary factors influence water, ion, and nutrient dynamics in the GIT.

6.8 Conclusions

Overall, the impact of dietary factors on chyme conditions and water fluxes in the gastrointestinal tract of freshwater rainbow trout was found to be minimal or absent. Indeed, regardless of diet composition or feeding level, the influx of water into the stomach is counteracted in the proximal intestine. Additionally, the net water flux along the gastrointestinal tract remains comparable across various dietary factors in freshwater fish. These findings indicate that freshwater fish rely on endogenous secretions in the gastrointestinal tract for water absorption and limit their water intake to

maintain osmoregulation. In seawater, however, the ingested water serves for osmoregulation rather than digestion. For instance, when smolt Atlantic salmon are fed a commercial diet, ingested seawater bypasses the stomach and enters the proximal intestine, where it is absorbed for osmoregulation. Additionally, dietary factors influence water fluxes in seawater fish. Indeed, when fed a low dietary electrolyte balance (dEB) diet, drinking rate increases in seawater-adapted Atlantic salmon.

Based on these findings, the following main conclusions can be drawn from the present thesis in which fish were fed dry pellet:

- In freshwater, the role of the gastrointestinal tract in osmoregulation is minimal compared to seawater, and it is primarily dedicated to dietary ion uptake.
- In seawater, fish drink for osmoregulation rather than for digestive purposes.
- dEB interferes with osmoregulation (water fluxes) in the gastrointestinal tract of Atlantic salmon in seawater but not in freshwater.

APPENDICES

References

Summary

Acknowledgements

Curriculum vitae

List of publications

WIAS Training and Supervision Plan (TSP)

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Summary

In response to the growing demand for food and the need to reduce food waste, aquaculture is transitioning towards the use of alternative ingredients, such as plant-based substitutes. This shift aims to minimize competition with human food consumption and offset the declining availability of fish meal. However, replacing fish meal with plant-based ingredients can compromise the nutritional quality and digestibility of the diet, potentially affecting water and ion balance (osmoregulation) along the gastrointestinal tract (GIT) of fish. Osmoregulation involves the secretion of fluids that alter water fluxes along the GIT, maintaining ion balance and ensuring proper digestive function. Previous studies on osmoregulation have typically focused on starved fish. However, it is now recognized that feeding can influence osmoregulatory processes in the GIT during digestion. While the interplay between diet and osmoregulation remains a relatively unexplored area, understanding this relationship is crucial for optimizing feed formulation, fish growth performance and welfare. This research aimed to investigate the impact of dietary factors on GIT osmoregulation and digestion in two salmonids fish species: rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*).

In **Chapter 2** and **3**, rainbow trout were fed diets with contrasting dietary electrolyte balance (dEB), buffering capacity ($CaCO_3$), and macronutrient composition (protein-to-energy ratio) under freshwater conditions (0 ppt). The results indicated that dietary factors had minimal or no effect on osmoregulation (water fluxes) and digestion kinetics (nutrient fluxes) in the GIT of freshwater trout. Furthermore, regardless of dietary factors, water influx into the stomach is counteracted in the proximal intestine, implying that freshwater fish tend to minimize the water entering the GIT.

Subsequently, the interaction between feeding and water salinity was examined in smolt Atlantic salmon. In **Chapter 4**, fish were fed a commercial

diet at increasing water salinities (0, 10, 20, 30 ppt). Water drinking rate increased with increasing water salinity, while water influx in the stomach remained consistent across salinities. This suggests that ingested water bypassed the stomach and directly entered the proximal intestine, for osmoregulatory purposes rather than chyme liquefaction. These findings highlight the dual function of the GIT in seawater fish, which serves both osmoregulatory and digestive roles. In contrast, the primary function of the GIT in freshwater fish is digestion, with minimal osmoregulatory involvement, primarily limited to ion uptake. Additionally, water salinity influences protein absorption along the intestine, with seawater fish exhibiting more distal absorption and freshwater fish showing more proximal absorption.

In **Chapter 5**, smolt Atlantic salmon reared in freshwater and seawater were fed diets with contrasting dEB levels to assess the impact of an unbalanced electrolyte diet on osmoregulation and digestion. While the diet had minimal effects on water and nutrient kinetics in freshwater fish, it significantly influenced drinking rate and water fluxes (and absorption) in the GIT of seawater-adapted salmon. The low dEB diet increased drinking rate and the overall magnitude of water fluxes across the GIT of seawater-adapted salmon. This suggests that dietary composition differentially affects osmoregulation and digestion in fish depending on water salinity. This finding further emphasizes the dual role of the GIT, particularly in seawater fish. It is possible that the low dEB diet disrupted physiological processes in the GIT, prompting the fish to drink more water to compensate.

Finally, **Chapter 6** provides a comparative analysis, comparing the findings of the current study to those available in literature. Furthermore, the implications of these findings for aquaculture are discussed.

Based on these findings, the following main conclusions can be drawn from the present thesis in which fish were fed dry pellet:

- In freshwater, the role of the gastrointestinal tract in osmoregulation is minimal compared to seawater, and it is primarily dedicated to dietary ion uptake.
- In seawater, fish drink for osmoregulation rather than for digestive purposes.
- dEB interferes with osmoregulation (water fluxes) in the gastrointestinal tract of Atlantic salmon in seawater but not in freshwater.

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About the author

Elisa Ciavoni was born on the 28th of January 1994 in Rome, Italy. After completing her secondary education, she studied Natural Science (BSc) at the University of Rome La Sapienza. There, she earned enhanced knowledge on animal anatomy, zoology, physiology, ecology, ethology, botany, and earth sciences. During her BSc, she spent five months at Sea Lion Island (Falkland Islands) where she conducted an experimental thesis on behavioral mating system of male and female southern elephant seals (*Mirounga leonina*).
Later, she started her specialization in Marine Biology and Ecology (MSc) at the University of Tuscia (Italy). At that moment, she was introduced to aquaculture and developed a strong interest in fish nutrition. Therefore, for her MSc thesis, she moved to the University of Udine (Italy), where she worked on a research aimed at studying the biological responses of carnivorous fish species to diet containing microalgae dried biomass as potential novel ingredients. Afterwards, she moved to the Netherlands through the Erasmus traineeship program within Wageningen University & Research, with the goal of learning further about fish nutrition and physiology. She participated in some PhD studies within the AFI group (department of Animal Science) before starting her own PhD project in September 2019.

During her PhD, Elisa focused on two important salmonid species for aquaculture: rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). She carried out her experiments at Wageningen University in the Netherlands and the Bergen Institute of Marine Research (IMR) in Norway. The study aims to improve understanding of the interplay between osmoregulation and digestion in the gastrointestinal tracts of rainbow trout and Atlantic salmon under both freshwater and marine settings. This could help enhance the overall growth and wellbeing of these fish through improved feed formulation.

List of publications

Ciavoni, E., Schrama, J. W, Sæle, Ø. & Philip, A. J. P. (2024). Dietary electrolyte imbalance alters drinking rate and gastrointestinal tract water fluxes of Atlantic salmon (*Salmo salar*) smolt in seawater. *Aquaculture*, 582, 740685.
https://doi.org/10.1016/j.aquaculture.2024.740685

Ciavoni, E., Schrama, J. W., Radhakrishnan, G., Sæle, Ø., & Philip, A. J. P. (2024). Salinity induced changes in the progression of water, ion and nutrient fluxes along the gastrointestinal tract of Atlantic salmon smolt (*Salmo salar*). *Aquaculture*, *580*, 740331. https://doi.org/10.1016/j.aquaculture.2023.740331

Ciavoni, E., Nederlof, M., Rooijakkers, J., Schrama, J. W., & Philip, A. J. P. (2023). Effect of dietary macronutrient composition and buffering capacity on chyme characteristics and digestion kinetics in the gastrointestinal tract of freshwater rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *574*, 739674.
https://doi.org/10.1016/j.aquaculture.2023.739674

Ciavoni, E., Maas, R. M., Koppelaars, M., Sæle, Ø., Schrama, J. W., & Philip, A. J. P. (2023). Effect of dietary electrolyte balance on the interplay between water fluxes and digestive functioning along the gastrointestinal tract of freshwater rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, *563*, 738928.
https://doi.org/10.1016/j.aquaculture.2022.738928

Elesho, F. E., Kröckel, S., Ciavoni, E., Sutter, D. A., Verreth, J. A., & Schrama, J. W. (2021). Effect of feeding frequency on performance, nutrient digestibility, energy and nitrogen balances in juvenile African catfish (*Clarias gariepinus*) fed diets with two levels of crystalline methionine. *Animal Feed Science and Technology*, *281*, 115098. https://doi.org/10.1016/j.anif eedsci.2021.115098

WIAS Training and Supervision Plan (TSP)

EDUCATION AND TRAINING			Year	ECTS
The Basic Package				
WIAS Introduction Day			2019	
Course on philosophy of science and/or ethics			2019	
Subtotal				**2**
Disciplinary Competences				
Writing the research proposal			2019	
Species-specific course on Fish			2019	
AQUAEXECEL Fish Nutrition and Feeding			2019	
WIAS/PE&RC advanced statistics course Design of Experiments			2019	
Laboratory Animal Science: Design and Ethics in Animal Experimentation			2020	
Fish Nutrition workshop			2021	
Subtotal				**13**
Professional Competences				
The Essential of Scientific Writing and Presenting			2019	
Presenting with Impact			2019	
Scientific Artwork - Vector Graphics and Images			2020	
Project and Time Management			2020	
Scientific Writing			2021	
Un-box your PhD			2020	
Introduction to LaTex			2021	
Intensive Writing Week			2022	
Subtotal				**8**
Presentation Skills				
WIAS Science Day	Poster presentation	Lunteren, The Netherlands	2020	
EAS Conference	Oral presentation	Funchal, Madeira	2021	
ISFNF Fin Fish Nutrition Conference	Poster presentation	Sorrento, Italy	2022	
Subtotal				**3**

Teaching competences		
Supervising MSc thesis students	2020	
Supervising MSc thesis students	2021	
Supervising MSc thesis students	2021	
Subtotal		**6**
Education and Training Total (minimum 30 credits)*		**32**
**One ECTS credit equals a studyload of approximately 28 hours*		

Colophon

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