# Effect of changing $CO_2$ levels and elevated salt content in feed for prevalence of nephrocalcinosis in Atlantic salmon (*Salmo salar* L.).

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# Abstract

This study explores the effects of rapidly changing water $CO_2$ levels and elevated salt content in feed as potential risk factors for development of nephrocalcinosis in Atlantic salmon (*Salmo salar L.*). Nephrocalcinosis is one of the main challenges in land-based FW production of Atlantic salmon and has been linked to accumulation of $CO_2$ in production water. Elevated $CO_2$ levels disturb acid-base regulation in fish, which is compensated by retaining bicarbonate ($HCO_3^-$). During aquaculture handling procedures, fish may be transferred from rearing tanks with high accumulation of $CO_2$ to new tanks with low $CO_2$ levels. This can trigger respiratory alkalosis and a rise in pH, because $PCO_2$ is rapidly flushed out across the gills, while downregulation of $HCO_3^-$ can take up to 24 hours.

This was studied by subjecting Atlantic salmon to different salt diets (low, medium, high), and three cycles of sustained high $CO_2$ (30 mg/L) levels followed by a rapid drop to control (<8 mg/L) levels. Meanwhile, acid-base parameters, ion concentrations, hematological parameters, growth, $Na^+/K^+$ ATP-ase (NKA) enzyme activity and prevalence of nephrocalcinosis were measured. The results indicate that high $CO_2$ increased the risk of developing nephrocalcinosis, and that prevalence increased with higher salt content in diets. Bicarbonate ($HCO_3^-$) was accumulated in response to high $CO_2$ levels and remained elevated five hours after dropping to control levels. Urinary catheterization revealed a rise in pH after dropping $CO_2$ levels, likely due to retained $HCO_3^-$. Changing $CO_2$ levels affected regulation of several ions, with multiple deviations from observations in previous research, indicating that knowledge is limited on the effects of acid-base disturbances and salt feeds on osmoregulatory mechanisms. Precipitation rate of kidney stones typically found in salmonids are described to increase with increasing pH. However, prevalence of nephrocalcinosis was high both before and after dropping $CO_2$ levels, and prevalence could not be attributed to the drop alone.

# Abbreviations

ADPS - N-Ethyl-N-(3-sulfopropyl)-m-anisidine

AM – Assay mixture

ATP – Adenosine triphosphate

BCG – Bromocresol green

BR – Biomass reduction

CA – Carbonic anhydrase

CF – Condition factor

ClC-K – basolateral specific $Cl^-$ channel

FE – Fractional excretion

FT – Flow through

FW – Freshwater

GEDTA - Glycoletherdiamine-tetraacetic acid

GFR – Glomerular filtration rate

Hb - Hemoglobin

Hct - Hematocrit

IB - Imidazole

LDH – Lactic dehydrogenase

MCHC – Mean corpuscular hemoglobin concentration

MDH – Malate hydrogenase

NADH - β-Nicotinamide adenine dinuclotide

NCC – $Na^+/Cl^-$ co-transporter

NHE – $Na^+/H^+$ exchanger

NKA – $Na^+/K^+$ ATP-ase

NKCC2 - $Na^+/K^+/Cl^-$ co-transporter

PEP – Phosphenylpyruvate

PEPC - Phosphoenolpyruvate carboxylase

PIT – Passive integrated transponder

PK – Pyruvate kinase

RAS – Recirculating aquaculture system

RBC – Red blood cell

SEM – Standard error of mean

SGR – Specific growth rate

SW – Seawater

TAN – Total ammonia nitrogen

TJ – Tight junction

UFR – Urine flow rate

UP – Urine-plasma

4AAP - 4-aminoantipyrine

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# 1. Introduction

Norwegian aquaculture had its breakthrough in the 1970's by successfully farming salmonids in floating net pens and has since become the world's leading producer of farmed Atlantic salmon (*Salmo salar*) (Nærings- og fiskeridepartementet, 2021). In 2024, Norway exported almost 1.6 million tons of farmed Atlantic salmon yielding a landing value of over 103.5 billion Norwegian kroner and contributing to approximately 70 percent of the country's total aquaculture exports (Norges sjømatråd, 2024; Fiskeridirektoratet, 2025). The aquaculture industry has become the second largest export industry in Norway and is one of the most important industries in Norwegian rural areas (Olaussen, 2018). This rapid increase in production was facilitated by Norway's long coastline and geographical advantage, which provide ideal farming environments for cold water species like Atlantic salmon, and also advancements in production methods, technology and broader biological knowledge (Kumar and and Engle, 2016; Fiskeridirektoratet, 2024b). A critical part of this success has been the refinement of land-based production of juvenile Atlantic salmon in freshwater (FW) facilities until they are ready for transfer to open net pens in seawater (SW). The development of photoperiod manipulation, improved water treatment systems, and vaccination protocols have enabled higher production for a year-round supply, while improving smolt survival and performance after seawater transfer (Duncan *et al.*, 1998; Bergheim *et al.*, 2009).

Norway produced 420 million salmon smolts in 2023, while the number of active smolt production licenses was reduced from 213 in 2010 to 186 in 2023 (Fiskeridirektoratet, 2024a). Production volume of juvenile Atlantic salmon has increased due to the combination of improved production capacity for each production site and accelerated production intensity. Producers aim to maximize biomass output per facility by increasing stocking density, reducing generation times and enhancing environmental control. Production of juvenile Atlantic salmon has traditionally taken place in land-based flowthrough (FT) facilities, but implementation of partial reuse systems and recirculating aquaculture systems (RAS) is increasing (Bergheim *et al.*, 2009) as water usage is the main limiting factor for land-based aquaculture (Blancheton *et al.*, 2007). Poor water quality as a result of stocking density are counteracted by implementing technologies like oxygen injection and carbon dioxide degassing (Kristensen *et al.*, 2012), while manipulation of temperature and photoperiod reduces rearing time and allows for earlier transfer to SW (Lysfjord, Jobling and Solberg, 2004).

As smolt producers are implementing technologies to limit water usage, they can reduce the flow rate and exchange of water in rearing tanks. This can cause carbon dioxide ($CO_2$) and total ammonia nitrogen (TAN) to build up and become a limiting factor, as well as causing problems with fish welfare (Fivelstad *et al.*, 1999; Blancheton *et al.*, 2007; Good *et al.*, 2018). Both acute and long-term exposure to elevated $CO_2$ levels is associated with reduced feed intake, growth, condition factor, stress recovery rate, and increased prevalence of nephrocalcinosis (Good *et al.*, 2018; Khan, Johansen and Skov, 2018). These effects of intensified smolt production is reported by the Norwegian Veterinary Institute (Moldal *et al.*, 2025), rating poor water quality and nephrocalcinosis as some of the major problems regarding mortality, fish health and welfare. This highlights the biological challenges associated with intensive aquaculture conditions, that deviate from the natural environment of Atlantic salmon.

## 1.1 Lifecycle

The Atlantic salmon is an euryhaline species that can adapt to a wide range of salinities, and is characterized by an anadromous lifecycle where they hatch in FW rivers before they migrate into SW to forage and mature (Hansen and Quinn, 1998). In FW rivers, adult salmon spawn and bury their fertilized eggs in the gravel streambeds in the fall or winter, before hatchlings emerge as fry in the spring. Juvenile salmon will typically spend 2-4 years in the river as parr, before they undergo smoltification to adapt to saline environments and seaward migration as smolts. During smoltification, they gradually lose their parr marks, become more streamlined and silvery in color, exhibit downstream migratory and schooling behavior, and preparatory osmoregulatory changes in gill, kidney and intestine allow for SW tolerance (Nilsen *et al.*, 2008; Hoar, 2024). The post-smolts then migrate to feeding grounds in the open ocean to forage for one to several years, until they eventually, as maturing adults, return to their native river to spawn.

## 1.2 Ion regulation

Ion regulation is the process of maintaining ion concentrations in body fluids within a range necessary for survival in an organism. Internal ion homeostasis can be affected by environmental or physical conditions but is maintained by regulation of ion and water fluxes between plasma and other body fluids and the external environment (Zimmer and Perry, 2022). In fish, the gill is considered the main organ for ion regulation, but the kidney and intestine are also ion transporting organs (Evans, Piermarini and Choe, 2005). Chloride cells, also known as ionocytes, are the main site for ion uptake in FW and secretion in SW. Fish in FW environments are hyperosmotic and depends on FW ionocytes for active ion uptake (influx) to counteract

passive diffusion (efflux) of ions to the less saline environment, while fish in SW are hypoosmotic and depend on SW ionocytes for active secretion (efflux) of ions to counteract passive uptake (influx) from the more saline environment. Anadromous Atlantic salmon moves between FW and SW environments during its lifecycle and depends on both FW and SW ionocytes when moving between environments with different salinities.

### 1.2.1 The $Na^+/K^+$-ATPase pump

The $Na^+/K^+$-ATPase (NKA) enzyme pump is a membrane protein present in all animal cells and is responsible for establishing and maintaining an electrochemical gradient across cell membranes that is used as an energy source for secondary transport of ions and other solutes against their concentration gradients (Skou and Esmann, 1992; McCormick, Regish and Christensen, 2009). NKA actively transports three sodium ions out of the cell and two potassium ions into the cell for every ATP consumed, and despite passive leakage of ions the NKA maintains the resting membrane potential using a large portion of the cell's energy at rest (Skou and Esmann, 1992). Maintaining the membrane potential enables ion transport against its gradients and thus ensures normal functioning of physiological processes. In teleost fish, the NKA enzyme is especially abundant in ion transporting organs like the gills, kidney and intestine, and are therefore important for both ion regulation and acid-base balance (McCormick, Regish and Christensen, 2009; Takvam *et al.*, 2024).

### 1.2.2 Ion regulation in the gills

The gills are the primary site for ion exchange in FW, where they actively take up sodium and chloride from the environment. It is made up of primary and secondary filaments lined with epithelial cells with different structural and physiological functions, where ions can move across the gills either through (transcellular) or between cells (paracellular). Transcellular ion uptake from the environment is regulated by ionocytes that contain specialized transporter proteins in the apical (water facing) and basolateral (blood-facing) membranes. Paracellular transport is regulated by structural complexes called tight junctions (TJ), where ions can move between adjacent epithelial cells. Depending on the environment the fish can regulate the surface area of the gills to optimize for either gas exchange or ion diffusion. Ion diffusion is proportional to surface area and diffusion distance, so optimizing for ion diffusion in FW will lead to a simultaneous reduction in gas exchange, and this trade-off is called the osmorespiratory compromise (Wood and Eom, 2021).

While fish are in FW, the ion transporting ionocytes gain an influx of ions firstly by absorption through the apical membrane. Apical sodium uptake is mainly a result of three different pathways called the sodium-hydrogen exchanger (NHE), sodium uptake mediated by apical $H^+$-ATPase via cation channels, or by sodium-chloride co-transporters (NCC), that all utilize the electrochemical gradient established by NKA. NKA is responsible for basolateral transport of sodium out of the cell and potassium into the cell, making the inside of the ionocytes low in sodium ions and highly negative. The two main mechanisms for chloride uptake in FW are $Cl^-$ /$HCO_3^-$ exchange (anion exchanger) and NCC, while basolateral transport of chloride into the blood plasma occurs by passive transport via chloride channels. The rate of ion uptake in the gills can be regulated by changing the functional surface area of ionocytes by expanding or contracting adjacent pavement cells, and thereby altering the surface area exposed to the external environment (Goss, Laurent and Perry, 1992; Wilson and Laurent, 2002).

### 1.2.3 Ion regulation in the kidney

The kidney is the main site for reabsorption of solutes and excretion of excess water for fish in FW environments (Perry *et al.*, 2003). The kidney runs dorsally along the abdominal cavity and ventral to the spine, spanning from the head to the urogenital opening. The kidney consists of the head kidney anteriorly serving as a hematopoietic, lymphatic and endocrine organ, while the mid and posterior kidney are rich in nephrons and is responsible for blood filtration and urine production (Resende *et al.*, 2010). The nephrons in euryhaline fish like Atlantic salmon are divided into several sections: glomerulus, proximal tubule 1 and 2, distal tubule, collecting tubule and collecting duct. The glomerulus is the first section that consists of a complex network of capillaries enclosed by podocytes where blood is filtered, and a filtrate called pre-urine is formed in the Bowman's capsule (Brown, Taylor and Gray, 1983). During filtration, only small enough solutes can pass through to the Bowman's capsule, while proteins and red blood cells (RBC) do not. The pre-urine is almost identical to plasma, but has no proteins, and it is subject to reabsorption and secretion processes as it goes through the proximal and distal tubules, before it ends in the collecting tubule and duct. The collecting ducts of different nephrons merge eventually forming the urinary bladder, where urine is stored until it is discharged through the urinary papilla (Curtis and Wood, 1991).

When the pre-urine enters proximal tubule I, organic acids are secreted while sodium and chloride are reabsorbed together with some water (Takvam, Wood, *et al.*, 2021). The proximal tubule II is the main site for reabsorption of divalent ions like magnesium ($Mg^{2+}$), calcium ($Ca^{2+}$) and sulphate ($SO_4^{2-}$), in addition to sodium, chloride, potassium and bicarbonate which

are also reabsorbed. The distal tubule and following sections have impermeable features which allows for reabsorption of sodium and chloride with minimal osmotic reabsorption of water, creating highly dilute urine. As for the ion transporters in the gill ionocytes, the NKA in nephron tubule cells creates the electrochemical gradient utilized for secondary reabsorption of ions, water and other solutes as well as secretion in the nephrons. The high glomerular filtration rate (GFR: 4-16 ml/Kg/h) of plasma (280 mOsm) and urinary flow rate (UFR: 1-6 ml/Kg/h) of diluted urine (20-50 mOsm) ensures reabsorption of ions and excretion of water. For SW acclimated fish, these mechanisms are reversed with a low GRF (0.2-1.5 ml/Kg/h) of plasma (320 mOsm) and low UFR (0.2-0.3 ml/Kg/h) creating highly concentrated urine (300 mOsm) rich in $Ca^{2+}$ and $Mg^{2+}$, which is important for conserving water while excreting divalent ions.

### 1.2.4 Ion regulation in the intestine

The intestine is the main organ for nutrient uptake from feed, but also serve an important role in fluid uptake, and is therefore important for ion regulation (Sundell and Sundh, 2012). Intestinal NKA is the main driving force for fluid and secondary ion and nutrient uptake, and is evenly distributed along the basolateral membrane of epithelial enterocytes lining the gastrointestinal tract, with a higher NKA concentration in the distal intestine compared to the posterior (Sundh *et al.*, 2014; Takvam *et al.*, 2024). In FW, the intestine absorbs sodium, chloride, calcium and magnesium ions from feed (Becker *et al.*, 2012; Sundell and Sundh, 2012). Fish in FW will avoid drinking, but some water will enter when food is ingested and will be absorbed together with ions and nutrients.

## 1.3 Acid-Base regulation

Acid-base regulation is the process of maintaining constant pH in intra- and extracellular fluids, and is essential for normal molecular, cellular and whole animal performance (Brauner *et al.*, 2019; Takvam *et al.*, 2023). Fish, like other animals, produce $CO_2$ during aerobic metabolism, and this is a weak acid that can quickly disturb the acid-base balance. However, $CO_2$ is effectively transported out of the blood across the gills because its solubility is almost 30 times higher than oxygen ($O_2$) and fish needs a high gill ventilation volume for sufficient uptake of $O_2$ (Heisler, 1993). As a result, the arterial partial pressure of $CO_2$ ($P_aCO_2$) values in fish are similar to the environmental $PCO_2$. Blood pH at any temperature and ionic strength is determined by the balance of bicarbonate concentration and $P_aCO_2$, and a change in one of these factors without a corresponding change in the other will cause a disturbance in acid-base homeostasis (Zimmer and Perry, 2022). If the $P_aCO_2$ is higher than the bicarbonate concentration, it is considered respiratory acidosis, and if the bicarbonate concentration is

higher than the $P_aCO_2$, it is considered respiratory alkalosis. In order for fish to maintain normal pH levels in the blood, they regulate acid-base equivalents ($H^+$, $NH_4^+$, $OH^-$ and $HCO_3^-$), ammonia and phosphate through cellular buffering, renal excretion and altering electroneutral exchanges of cations and anions across the gill (Hōbe, Wood and Wheatly, 1984; Takvam *et al.*, 2023). When acid-base homeostasis is disturbed, excretion of $H^+$ and $NH_4^+$ represent acid equivalent excretion, while excretion of $OH^-$ and $HCO_3^-$ represent basic equivalent excretion. Despite the respiratory gasses $CO_2$ being acidifying and $NH_3$ being alkalizing, they are not considered to be acid-base equivalents as the normal pH of fish is approximately 7.2-8.2, and is much higher than the pK of $CO_2$-$HCO_3^-$ interconversion, and much lower than the pK of the $NH_4^+$-$NH_3$ interconversion, with $HCO_3^-$ and $NH_4^+$ making up over 95% of the bodily concentration (Randall and Wright, 1989). Movement of these charged acid-base equivalents needs facilitation by transporters or channels, while the uncharged gasses $CO_2$ and $NH_3$ can easily move between cell membranes.

The gill is considered the main regulatory site for acid-base balance in teleost fishes, handling approximately 90-95% of the total acid-base regulation (Takvam *et al.*, 2023). When metabolic $CO_2$ enters the blood, it becomes rapidly hydrated to $HCO_3^-$ by the enzyme carbonic anhydrase (CA) to ensure a typical blood pH of 7.9 and $HCO_3^-$ levels of 8 mM (Hōbe, Wood and Wheatly, 1984; Brauner *et al.*, 2019). Once blood reaches the gills another CA enzyme dehydrates $HCO_3^-$ back to $CO_2$, which allows rapid diffusion of $CO_2$ across the gill epithelium. Protons ($H^+$) are secreted or taken up via an $Na^+/H^+$-exchanger (NHE) or pumped out by an H-ATPase enzyme, while excretion or uptake of $HCO_3^-$ is achieved by the $Cl^-/HCO_3^-$ exchanger (anion exchanger) (Zimmer and Perry, 2022). When acid-base regulation is disturbed the exchange rate of $Na^+$ for $H^+$ and $Cl^-$ for $HCO_3^-$ are increased, resulting in elevated plasma $Na^+$ levels and decreased plasma $Cl^-$ levels during respiratory acidosis.

The kidney has a minor, but often overlooked, role in acid-base regulation (Takvam *et al.*, 2023). In FW acclimated rainbow trout (*Oncorhynchus mykiss*, previously *Salmo gairdneri)*, the kidney accounts for 7% of the secretion of acid equivalents during hyperoxia and 16% during hypercapnia (Wheatly, Hōbe and Wood, 1984; Perry, Malone and Ewing, 1987). As the pre-urine flows through the tubules, $HCO_3^-$ is effectively recovered and returned to the blood, resulting in normal urine concentrations of $HCO_3^-$ around 1-2 mM and pH of 7.3. As in the gills, this is achieved by CA facilitating conversion of $HCO_3^-$ to $CO_2$, which then diffuse from the tubule lumen and into the cells in the nephrons where it gets converted back to $HCO_3^-$ and transported to the blood by a $Na^+$-$HCO_3^-$ co-transporter. Additionally, phosphate ($PO_4^{3-}$) and

ammonia ($NH_3$) are two important urinary buffers that are important during respiratory (Wheatly, Hōbe and Wood, 1984) and metabolic (Wood, Milligan and Walsh, 1999) acidosis to counter the buildup of $H^+$ in the pre-urine.

The intestine has a more pronounced role in acid-base regulation for SW acclimated teleosts than in teleosts acclimated to FW (Wilson, Wilson and Grosell, 2002; Brauner *et al.*, 2019). In SW acclimated teleosts the intestine is important for water uptake and the intestinal osmolality is lowered by secreting $HCO_3^-$ into the lumen, which bind $Ca^{2+}$ and precipitate out as calcium carbonate, $CaCO_3$ (Brauner *et al.*, 2019). The role of the intestine in FW acclimated teleosts are minor and will thus not be addressed in detail in this thesis.

## 1.4 Hematology

Plasma and red blood cells (RBC) are the main contents of fish blood, and are responsible for transporting solutes and $O_2$ to cells of the body through the circulatory system, while waste products like $CO_2$ are transported away (Kryvi and Poppe, 2021). Plasma of vertebrates is mostly water (~90%) and some solutes (~10%) in the form of various proteins, organic compounds and ions that help maintain blood pH (Mathew, Sankar and Varacallo, 2025). RBC, or erythrocytes, are nucleated cells that make up 20-40% of the blood volume (Hematocrit) and contain hemoglobin, a protein that binds to $O_2$ and transports it from the gills to the rest of the body (Souza and Bonilla-Rodriguez, 2007). The oxygen carrying capacity is therefore determined by hemoglobin concentration within each cell measured as mean corpuscular hemoglobin concentration (MCHC). However, the oxygen binding affinity of hemoglobin in teleost fishes is dependent on blood pH, where binding affinity decreases with a decrease in pH, a mechanism described as the Root Effect (Brittain, 1987).

## 1.5 Nephrocalcinosis

Nephrocalcinosis has become one of the main challenges in land-based FW production of juvenile Atlantic salmon and is an important welfare indicator regarding water quality or rearing methods (Moldal *et al.*, 2025). It is characterized by precipitation of minerals salts within the kidney tubules and collecting ducts, which can be visually observed. However, mineral deposits can be too small for macroscopic observation by the naked eye. The condition may cause dilation of tubules and collecting ducts, and eventually degeneration and necrosis in the epithelium of affected structures (Klykken *et al.*, 2022). Additionally, the hematopoietic, immunological and endocrine tissues of the kidney may also be damaged causing reduced immune function and reduced production of RBCs (Klykken *et al.*, 2022; Moldal *et al.*, 2025).

The composition of mineral deposits is mainly a combination of phosphate, calcium, magnesium and carbonate, with amorphous carbonate apatite ($Ca_{10}(PO_4)_6CO_3$) and struvite ($MgNH_4PO_4\ 6H_2O$) being the most prevalent (Klykken *et al.*, 2022). These mineral stones are found in both humans and salmonids and the rate of precipitation increases with increasing pH, with amorphous carbonate apatite found to precipitate at a pH of 6.8 or higher, while struvite has been found to precipitate at pH of 7.2 or higher (Olszynski, Prywer and Torzewska, 2015).

## 1.6 Causes of nephrocalcinosis

As mentioned earlier, land-based aquaculture rearing tanks are susceptible to $CO_2$ accumulation. Hypercapnia was early linked to nephrocalcinosis in salmonids (Smart *et al.*, 1979) and exposure to constant high $CO_2$ levels have been reported to coincide with increased prevalence of nephrocalcinosis (Fivelstad *et al.*, 1999, 2003; Hosfeld *et al.*, 2008) while other studies exposing Atlantic salmon to constant elevated $CO_2$ levels did not find nephrocalcinosis (Fivelstad *et al.*, 2007; Good *et al.*, 2018; Mota *et al.*, 2019). Thus, it appears that constant hypercapnia over time alone is not the only cause of this production disorder. Interestingly, Atlantic salmon exposed to both constant hypercapnia and hyperoxia developed higher prevalence of nephrocalcinosis than fish exposed to hypercapnia alone (Hosfeld *et al.*, 2008), probably due to higher build-up of $CO_2$ in the fish exposed to both hypercapnia and hyperoxia (Wood and Jackson, 1980; Hōbe, Wood and Wheatly, 1984). Salmonids exposed to hypercapnia and/or hyperoxia experience respiratory acidosis, resulting in a drop in blood pH that the fish compensates for by increasing the amount of inorganic phosphate, total ammonia (TAN) and acidic equivalents in the excreted urine, thereby augmenting the retention of $HCO_3^-$, which aids in normalizing blood pH (Wood and Jackson, 1980; Wheatly, Hōbe and Wood, 1984; Larsen and Jensen, 1997). Urine pH drops only slightly due to the buffering capacity of phosphate and ammonia and urinary concentrations of $Ca^{2+}$ and $Mg^{2+}$. While the concentration of $HCO_3^-$ may not be high in the final urine it might be very high in the tubular pre-urine and the combination of increased $PCO_2$, phosphate, ammonia, $Ca^{2+}$ and $Mg^{2+}$ concentrations might provide good conditions for precipitations of minerals in the kidney. Indeed, modern aquaculture often apply salinity in the water and commercial diets often include marine ingredients that make FW acclimated fish experience salt levels higher than what they would experience in their natural habitats. Furthermore, special transition diets with added $Ca^{2+}$, $Mg^{2+}$ and NaCl levels are also in use (Philip *et al.*, 2022; Ytrestøyl *et al.*, 2023). RAS facilities may also use calcium based buffers and the accumulation of specific ions in RAS, particularly $Ca^{2+}$,

$Mg^{2+}$ and phosphorous, are suggested to be risk factors for development of nephrocalcinosis (Klykken *et al.*, 2022; Takvam *et al.*, 2023).

Handling procedures like sorting and vaccination in modern aquaculture often involve moving fish from one tank to another. If fish are kept in a chronic state of respiratory acidosis due to hypercapnia when biomass increase in the tank, they will have compensated by accumulating $HCO_3^-$. When fish are rapidly moved from a tank with high $CO_2$ levels to a tank with low $CO_2$ levels, $PCO_2$ will quickly be flushed out across the gills, inducing respiratory alkalosis which rises blood pH and it may take up to 24 hours before $HCO_3^-$ are regulated back down (Hōbe, Wood and Wheatly, 1984; Wheatly, Hōbe and Wood, 1984; Perry, Malone and Ewing, 1987). This has been suggested to be the primary trigger for formation of kidney stones (Takvam *et al.*, 2023). Considering that pre-urine is essentially the same as plasma, it is likely that the pH of pre-urine in the kidney tubules also rises and creates favorable conditions for nucleation and precipitations of minerals, particularly when a drop in $PCO_2$ coincide with increased salt load via feed, water or accumulation of divalent calcium, magnesium and phosphorous in RAS, all of which are important components in kidney stones (Klykken *et al.*, 2022).

## 1.7 Aim of study

This study aims to increase our knowledge of how respiratory acidosis from hypercapnic rearing conditions followed by respiratory alkalosis induced from rapid change to low $CO_2$ levels, combined with salt loading in feed, affects the physiology of the main osmoregulatory organs (gills, kidney, intestine) and the prevalence of nephrocalcinosis in juvenile Atlantic salmon reared in FW RAS. First, I will investigate how the NKA enzyme activity changes in main osmoregulatory organs (Objective 1) and compare how the acid-base status is affected (Objective 2) before and after dropping $CO_2$ levels in the rearing tanks. Following this, I will investigate changes in renal handling of ions in response to acid-base perturbations and different salt loading in feed (Objective 3) and finally determine the effect of these factors on animal performance and prevalence of nephrocalcinosis (Objective 4).

**Objective 1: Determine NKA enzyme activity in the three main osmoregulatory organs before and after dropping $CO_2$ levels in rearing tanks.**

The NKA enzyme is abundant in the epithelial cells of the three main osmoregulatory organs in teleost fish and it is vital for ion regulation and acid-base balance as it generates the electrochemical transmembrane gradient utilized for secondary transport of ions and other solutes (Skou and Esmann, 1992; McCormick, Regish and Christensen, 2009; Takvam *et al.*,

2024). Osmoregulation and acid-base regulation is intimately connected (Zimmer and Perry, 2022) and uptake and secretion of acid-base equivalents would probably affect the NKA enzyme activity (Takvam *et al.*, 2023). I therefore expect an increase in NKA enzyme activity after dropping $CO_2$ levels because fish must compensate for rapid induction of respiratory alkalosis.

**Objective 2: Compare acid-base status before and after dropping $CO_2$ levels in rearing tanks.**

The internal $PCO_2$ in fish is affected by $CO_2$ produced from metabolism and from environmental $PCO_2$, and is compensated by regulating $HCO_3^-$ (Heisler, 1993). In land-based aquaculture, hypercapnic conditions are common, and rapid drops in water $CO_2$ levels occur when fish is moved to new tanks. When transitioning from hypercapnia to low $CO_2$ levels, $CO_2$ is effectively flushed out across the gills while adjustment of internal $HCO_3^-$ to normal levels can take up to 24 hours (Hōbe, Wood and Wheatly, 1984; Wheatly, Hōbe and Wood, 1984; Perry, Malone and Ewing, 1987). Simulating hypercapnia followed by a rapid drop in $CO_2$ levels when moved into new tanks, I expect the fish to experience high concentrations of $HCO_3^-$, elevated pH and low $PCO_2$ during the first hours after dropping $CO_2$ levels. After two weeks, I expect that the fish will have had the time to regulate and return to normal pH.

**Objective 3: Determine changes in renal handling of ions when acid-base regulation is disturbed, and the effect of different salt load in feeds.**

Perturbation of acid-base regulation is compensated by the regulation the exchange rate of $Na^+$ for $H^+$ and $Cl^-$ for $HCO_3^-$ (Zimmer and Perry, 2022; Takvam *et al.*, 2023). During respiratory alkalosis, salmonids compensate by increasing uptake of $H^+$ and secretion of $HCO_3^-$. Additionally, NaCl, phosphate and divalent ions like calcium and magnesium are often added to feed, making them abundantly available. These ions are used as urinary buffers to counter build-up of $H^+$ in pre-urine but are later reabsorbed (Hōbe, Wood and Wheatly, 1984; Wheatly, Hōbe and Wood, 1984; Wood, Wheatly and Hōbe, 1984). I predict that sodium levels will decrease while chloride levels increase five hours after dropping $CO_2$ levels, while phosphate, calcium and magnesium reabsorption is increased. Changes in reabsorption and secretion may also be linked to structural damage caused by kidney stones.

**Objective 4: Determine the effects of rearing environments with rapidly changing $CO_2$ levels and different salt loads in feed on animal performance and prevalence of nephrocalcinosis.**

Amorphous carbonate apatite and struvite are the most prevalent types of kidney stones in salmonids, and mainly consists of phosphate, calcium, magnesium and carbonate (Klykken *et al.*, 2022), and precipitation rate increases with increasing pH (Olszynski, Prywer and Torzewska, 2015). When $CO_2$ levels are dropped, which induces respiratory alkalosis and rises pH in the kidney, combined with increased salt load from feed, water, or accumulation in RAS, it creates favorable conditions for mineral precipitation (Takvam *et al.*, 2023). I therefore predict that a rapid drop in $CO_2$ levels and high calcium and magnesium diets will lead to higher prevalence of nephrocalcinosis compared to diets with lower salt loads. Additionally, as compensation for acid-base regulation disturbances requires increased energy expenditure, I predict a decrease in animal performance in relation to weight, specific growth rate and condition factor.

# 2. Material and methods

## 2.1 Fish material, experimental design and sampling methods

### 2.1.1 Fish material

Atlantic salmon (*Salmo salar* L.) used in this experiment was of Benchmark Genetics, Salten AS. Eyed eggs (387 day-degrees) arrived at Skretting Aquaculture Innovation Research station (Lerang, Norway) on the 30th of November 2023. Upon arrival, the eggs were incubated at a water temperature of 6°C, increasing to 7.3°C in December and January. On December 15th, 2% of the egg batch hatched, increasing to 5% on the 16th and to 30% by the 17th, with 100% of the egg batch being hatched by December 18th. Water temperature was increased to 11.1°C in February and fish was startfed from the 9th of February 2024. The feed used was Skretting Nutra Sprint EU 0.5 mm and Skretting Nutra Sprint, with gradually increased pellet size from 0.7, to 0.8 and 1.0 mm, until Skretting Olympic 1.5 mm pellet was introduced on 28th of May. On May 27th the fish were tagged with a passive integrated transponder (PIT) and transferred into 300 liters flow-through (FT) tanks with an average water temperature of 12.1±0.3°C. Continuous light was used from start feeding and until the end of experiment. Fish were fed in excess throughout the whole experiment.

### 2.1.2 Experimental design

On September 4th of 2024, a total of 1440 Atlantic salmon were randomly distributed into 12 single Recirculation Aquaculture Systems (RAS), resulting in 120 fish per tank and 240 fish per treatment. The fish were exposed to either fluctuating $CO_2$ levels (5-30 mg/L) or steady low

$CO_2$ levels (<8 mg/L), each of which were given 3 different dietary salt levels, creating 6 treatment groups (Table 1). The salts altered in the diet were a composition of calcium, phosphorus and magnesium. Calcium and magnesium contents were increased by approximately 1.5 times from low to medium, and from medium to high, while phosphorus contents were increased by approximately 1.2 times from low to medium, and from medium to high (see details in Table 2).

**Table 1:** Atlantic salmon (n= 120 per tank) were distributed into 12 single RAS tanks and exposed to either repeated fluctuating water $CO_2$ levels (High $CO_2$) or steady low $CO_2$ levels. Within each $CO_2$ treatment groups were fed with either low salt control diet (A), medium salt diet (B) or high salt diet (C), generating 6 experimental groups reared in single RAS tanks. Mean weight ± standard error of mean (SEM) of fish at the start of the experiment.

| ***Fluctuating water $CO_2$ levels (5-30 mg/L)*** | | | ***Steady low water $CO_2$ levels (<8 mg/L)*** | | |
|---|---|---|---|---|---|
| *Diet* | Tank number | Mean weight (g) ± sem | Diet | Tank number | Mean weight (g) ± sem |
| *A* | 1501 | 102 ± 1.2 | A | 1507 | 104 ± 1.2 |
| | 1503 | 104 ± 1.4 | | 1509 | 103 ± 1.3 |
| *B* | 1502 | 104 ± 1.1 | B | 1508 | 103 ± 1.1 |
| | 1506 | 104 ± 1.1 | | 1510 | 104 ± 1.3 |
| *C* | 1504 | 101 ± 1.2 | C | 1511 | 102 ± 1.3 |
| | 1505 | 106 ± 1.3 | | 1512 | 107 ± 2.1 |

Each circular RAS tank had a rearing volume of 1000 liter water and a flow rate of 75-liter freshwater (FW, 0-3 ppt) per hour to ensure a minimum of 80% $O_2$ saturation. The makeup water was 21 liters per hour. An automated dosing system added 5% NaOH buffer to maintain water pH at 7.2 in all experimental tanks. The tanks were covered with a lid to ensure minimal disturbance, and the ambient temperature outside of the tanks in the facility ranged from approximately 10-15°C.

Experimental diets were introduced after baseline sampling (T0) on the 4-5$^{th}$ of September, with all groups receiving the same treatment up until that point. Fish was split into groups after T0 and given a 3-week acclimatization period. Then, water $CO_2$ levels were gradually increased to 30 mg/L over 2 weeks by approximately 15 ppm per day from Monday to Friday each week, for the experimental $CO_2$ groups High-A, High-B and High-C. Experimental groups were held at peak $CO_2$ (30 mg/L) for 2 weeks until the first trial sampling, pre-drop (T1) on the 11-12$^{th}$ of November. After T1 on the 13-14$^{th}$ of November, water $CO_2$ levels were rapidly dropped for

the experimental groups, which reached control levels (<8 mg/L) after approximately 4-5 hours. The fish was sampled again for the second trial sampling, 5 hours post-drop (T2). All groups were kept at control levels for 2 weeks and sampled again for the third trial sampling, 2 weeks post-drop (T3) on the 28-29$^{th}$ of November. After T3, experimental groups went through 2 new cycles of high $CO_2$ levels lasting 7-9 weeks each. The $CO_2$ peak period lasted 3 and 4 weeks for the second and third cycle respectively.

A second urinary catheterization trial was run on the 3-14$^{th}$ of March 2025, at the end of the third cycle of high $CO_2$ levels while experimental groups were still at peak $CO_2$ (30mg/L). Four fish from each tank, meaning 8 from each group, were catheterized and placed in external catheterization chambers for 72 hours, including a 24-hour acclimatization period. After 48 hours in the chambers, water $CO_2$ levels were dropped by $CO_2$ stripping for all groups. During catheterization, urine was collected to monitor real time changes before, during and after the water $CO_2$ level drop for both the experimental and the control groups. After 72 hours, catheterized fish was sampled for the fourth trial sampling, catheterization sampling (T4).

In total there were 5 sampling times: T0 (physiology at baseline), T1 (physiology at peak $CO_2$), T2 (physiology at five hours after drop), T3 (physiology at two weeks after drop) and T4 (catheterization trial). Data from baseline sampling (T0) was not included due to a problem with water quality during sampling.

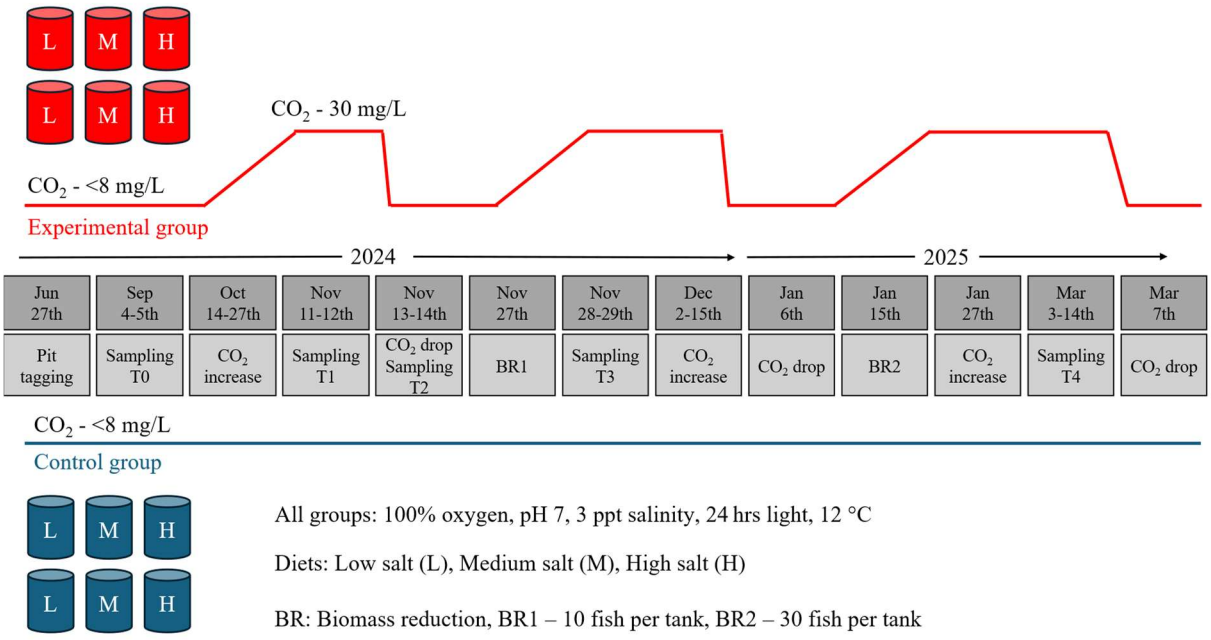


**Figure 1:** Shows the experimental timeline for the project and the $CO_2$ treatment and salt diets for the experimental and control groups. During $CO_2$ increase, water level $CO_2$ is slowly increased to 30 mg/L over two weeks for the

experimental group. At $CO_2$ drop, water $CO_2$ levels rapidly decreased, reaching <8 mg/L after approximately 4-5 hours. Fish were PIT-tagged prior to the start of the trial. Sampling T0 is the baseline sampling, sampling T1 is the pre-drop sampling, sampling T2 is the 5 hours post-drop sampling, sampling T3 is the two weeks post-drop sampling, and sampling T4 is the catheterization trial sampling after three cycles of increasing and dropping the $CO_2$ levels for the experimental groups. At biomass reduction 1 (BR1), 10 fish were removed from each tank. At biomass reduction 2 (BR2), 30 fish were removed from each tank.

### 2.1.3 Sampling protocols

Handling and sampling procedures can have negative effects on blood parameters. Thus, we followed a modified sampling procedure as described by Davison *et al.* (2023) to minimize potential adverse effects of sampling on blood and urine parameters in present study. Twenty-four hours prior to each sampling, 3 fish were dip-netted out of the experimental RAS tank and put in black circular 90-liter containers filled with 70-liter rearing water from the main tank. There were two buckets for each RAS tank, resulting in a total of 6 fish sampled from each RAS tank. For each bucket, an aquarium pump was placed in the main RAS tank, generating a flow rate of 2 liters per minute into the bucket while an outlet from the buckets allowed water to be gravity fed back into the main RAS tank, resulting in a retention time of 35 minutes for the water volume in the buckets.

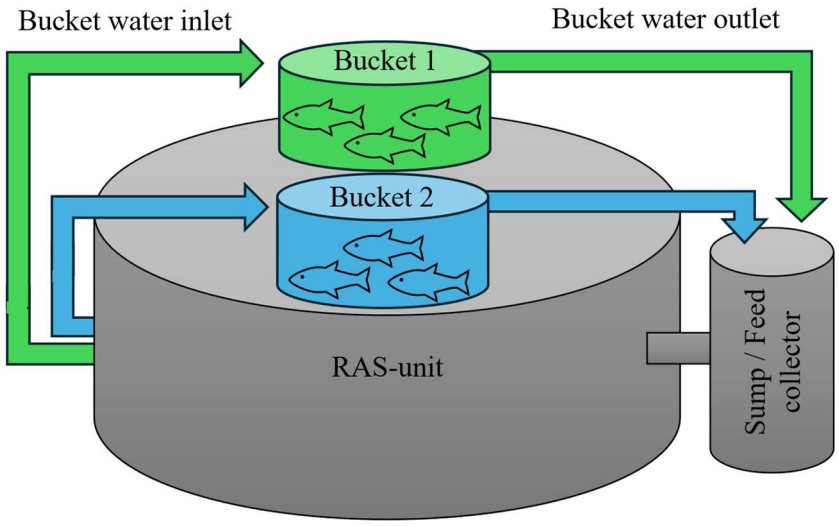


**Figure 2:** Shows the sampling buckets 1 and 2 on top of a RAS-unit, with 3 fish per bucket, for a total of 6 fish per RAS-unit. An aquarium pump supplied water to each sampling bucket from the RAS-unit with a flow rate of 2 L per minute, and the bucket outlet water returned to the sump/feed collector by gravity.

After 24 hours in the buckets, the water flow was stopped and fish were anesthetized with a non-lethal dose (50 mg/L) of tricaine methanesulphonate (MS-222, buffered to pH 7.1 with sodium hydroxide) while they were in their buckets, and then scoped out of the bucket using a container to keep the gills continuously submerged in water. The fish were immediately placed on a surgical table where the gills were continuously irrigated with aerated water (12°C, lethal doseMS-222: 100 mg/L) flow to ensure artificial ventilation, while urine and blood were sampled. An aquarium aeration pump with aeration stones was placed in the surgical table water, which created fine bubbles and a large surface area for oxygen diffusion into the water. This enabled reuse of surgical table water and easier maintenance of anesthesia levels.

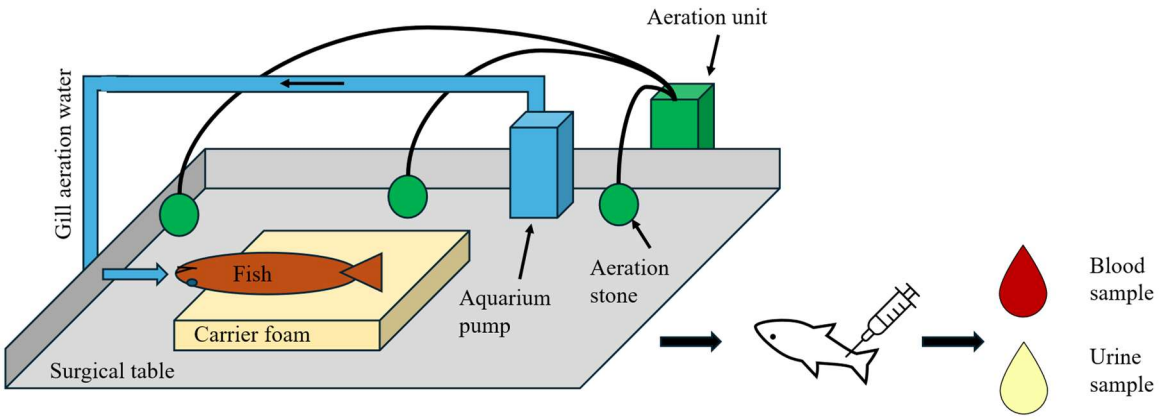


**Figure 3:** Shows the surgical table used during both physiological sampling and catheterization of fish. The fish was anesthetized prior to collection/surgery and was placed with the ventral side up on the carrier foam. The gills were supplied with aerated water through a tube going into the mouth, with water being circulated by an aquarium pump. An aeration unit with aeration stones supplied fresh air to the water on the surgical table.

Urine was collected using the urine spot sampling technique described by Curtis and Wood (1991), by gently pressing on the posterior part of the abdominal cavity so that urine could flow out of the urogenital opening and be collected using a 100 µl syringe (Hamilton 1710N NO. 1132491). Urine was transferred to 200 µl tubes and kept on ice until the pH was measured using a Thermo Scientific Orion 9810BN Micro pH Electrode (Prod. number 10655091) connected to a Mettler Toledo FiveGo$^{TM}$ handheld pH meter (Fisher, prod. number 15543380) as described by the manufacturer. Urine samples were then stored at -80 °C.

Blood was taken from the caudal artery/vein using a 2 ml heparinized syringe, and blood pH was immediately measured using a Hamilton BioTrode electrode (Prod. number 238140) and the Mettler Toledo FiveGo™ handheld pH meter. Disposable Hischmann™ hematocrit capillary glass was quickly filled with blood and centrifuged (3 minutes, 12000 g), and blood samples were added to Drabkin solution and kept on ice until hemoglobin levels were analyzed in the lab (section 2.3.2 for details).

The fish was scanned and the PIT-tag number read before body weight and fork length were recorded. The second gill arch on the left side were dissected out and divided into two pieces, one that was placed in a tube with 0.5 ml SEI buffer (250 mM sucrose, 10 mM ethylenediaminetetraacetic acid [EDTA], 50 mM imidazole), and the other without a buffer. A visual macro scoring scale from 1 to 4 for nephrocalcinosis in the kidney was then conducted according to Klykken *et al.* (2022), before two approximately 1 x 0.5 cm sections of the posterior part of the kidney were dissected out as outlined by Takvam, Denker, *et al.* (2021). One piece was placed in 0.5 ml SEI buffer and one in a tube without buffer. The anterior and distal intestine were dissected out, the faces were removed, and a longitudinal cut with scissors were performed. The intestinal tissue was placed with the serosa side facing down and the enterocytes was then scraped off before 1-1.4 mg of the scrapings were immersed in 100 µl intestinal SEIG buffer (300 mM sucrose, 45 mM Ethylenediaminetetraacetic acid (EDTA), 50mM imidazole, 200 mM glycine, 50 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid [EGTA]) with 1 tablet of Complete™ protease inhibitor cocktail (04693124001 Roche) for each 10mL batch of intestinal ice-cold SEIG buffer. All tissue samples and plasma aliquots were immediately frozen on dry ice and at -80°C until analyzed.

To measure urinary flow rates (UFR), internal catheters (Polyethylene 50, PE50, Intramedic®, Clay Adams, Parsipan, NJ, USA, prod. number 427411) were inserted into the urinary bladder of 8 fish per group, as outlined by Wood and Patrick (1994). Briefly, fish from each RAS tank were dip-netted out of the experimental RAS tanks and transferred to aerated buckets where they were anesthetized (MSS22: 60 mg/L) for approximately 4-8 minutes until their swallowing and ventilation reflex were suppressed. Anesthetized fish were transferred to the surgical table where gills were continuously irrigated with aerated water containing anesthesia (MS-222: 50 mg/L), ensuring artificial ventilation. A catheter filled with milliQ water was inserted 3-4 cm through the urogenital papilla into the urine bladder and secured by sewing five stiches posterior to the urogenital papilla. Two stitches were sewn anterior too, and three to the side of, the anal fin. Once the catheter was successfully secured, the fish was transferred into pre-made confined

chambers submerged into rectangular black buckets to restrict any movement and protect the catheter. The pre-made chambers were made by creating two separate longitudinal chambers (48,8 x 6 x 12 cm), on each side of a perforated grey rectangular shaped box (Clas Ohlson klappboks, 48,8 x 33,1 x 19 cm). The tip of the catheter ended in a falcon tube located 10 cm below the water level to ensure a sufficient siphoning effect and smooth continuous urine flow.

The collection of urine started after the first 24 hours of anesthesia and handling to minimize stress induced diuresis (Hunn and Willford, 1970). Thus, 24 hours after the catheter was successfully inserted, urine collection began. Urine was collected at 1, 2, 3, 4, 6, 8, 12 and 24 hours, with urine volume and pH recorded. Then, the water flow in the rectangular buckets with cannulated fish was put on a re-circulation loop by pumping water from the rectangular bucket and through a $CO_2$ stripper (diameter 18 cm, length 45 cm), decreasing the $CO_2$ levels in the water to <1 mg/L within 35 minutes. Urine was again collected at 1, 2, 3, 4, 6, 8, 12 and 24 hours, with urine volume and pH recorded. Twenty-four hours after $CO_2$ drop, blood and tissue samples from all catheterized fish were collected as described above. All urine and tissue samples were frozen and stored at -80°C until analyzed.

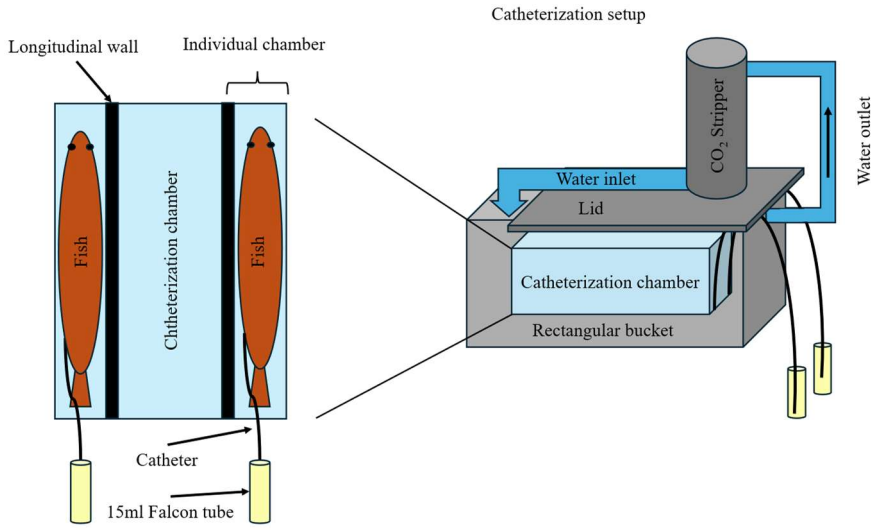


**Figure 4:** Shows the setup during catheterization sampling and the catheterization chamber. For each RAS-unit, 2 of these setups were used with 2 fish per catheterization chamber, for a total of 4 fish per RAS-unit. The catheterization chamber sat inside a rectangular bucket filled with water. One catheterization chamber was made up of a perforated box with longitudinal internal walls creating two individual chambers with one fish each. Catheters from each fish went out of the chamber and the rectangular bucket into 15 ml Falcon tubes. When dropping $CO_2$ levels during the catheterization sampling, water in the bucket was pumped to the $CO_2$ stripper by an aquarium pump with a flow rate of 2 liters per min. The water went back to the bucket from the $CO_2$ stripper creating a closed loop. A lid was placed on top of the bucket to stop the fish from jumping out, as well as serving as a stand for the $CO_2$ stripper.

### 2.1.4 Feed formulations

The feed used in this trail was produced by Skretting AI's feed technology plant in Stavanger, Norway. Three categories of diets were made: low salt (diet A), medium salt (diet B) and high salt (diet C), with the salt's calcium, phosphorus and magnesium content being altered in each diet (Table 2). In the low salt diet, the target concentration of calcium levels was approximately 10 000 mg/kg. The medium salt diet contained approximately 1.5 times the calcium concentration of the low salt diet, and the high salt diet appropriately 2 times the concentration of the low salt diet. For phosphorus the concentration levels in the low salt diet were approximately 11-12 000 mg/kg, with the medium and high salt diet containing approximately 1.2 and 1.5 times the concentration of the low salt diet respectively. For magnesium, the low salt diet had a concentration of approximately 2000 mg/kg, with the medium and high salt diets containing approximately 1.5 and 2 times the concentration of the low salt diet. The size of feed pellets changed during the trial due to fish size, and both a 2 mm and 3 mm pellet were given. Two versions of the same size 3 mm pellet were made for each diet, as feed was produced in batches.

**Table 2: Shows the proximate % content of fat, protein, moisture, ash and the measured amount of Calcium ($Ca^{2+}$), Phosphorous (P) and Magnesium ($Mg^{2+}$) in the low (A), medium (B) and high (C) salt diets.**

| | ***Proximate (%)*** | | | | ***Analyzed (mg/kg)*** | | | |
|---|---|---|---|---|---|---|---|---|
| ***Diet*** | **Fat** | **Protein** | **Moisture** | **Ash** | **P** | **$Ca^{2+}$** | **$Mg^{2+}$** | **Selenium** |
| *A1-2mm* | 26.7 | 47.6 | 6.5 | 6.6 | 11643 | 10714 | 2050 | 0.88 |
| *B1-2mm* | 26.7 | 48.7 | 6.9 | 8.0 | 13876 | 15559 | 3075 | 0.94 |
| *C1-2mm* | 27.0 | 48.3 | 7.0 | 10.2 | 17407 | 21308 | 4246 | 0.99 |
| *A2-3mm* | 26.9 | 47.4 | 6.8 | 6.6 | 11781 | 11220 | 2075 | 0.94 |
| *B2-3mm* | 27.3 | 48.1 | 6.8 | 7.9 | 13854 | 15427 | 3040 | 0.90 |
| *C2-3mm* | 26.9 | 49.4 | 6.8 | 10.2 | 17130 | 21361 | 4104 | 0.93 |
| *A3-3mm* | 26.6 | 46.9 | 7.4 | 6.6 | 11187 | 10331 | 1991 | 1.01 |
| *B3-3mm* | 26.7 | 47.9 | 6.7 | 7.9 | 13830 | 15402 | 3051 | 1.04 |
| *C3-3mm* | 26.3 | 48.7 | 6.8 | 10.4 | 17693 | 22183 | 4473 | 1.04 |

## 2.2 Calculations

### 2.2.1 Condition factor

Fulton's conditioning factor was calculated using this formula:

$$CF = \frac{W * 100}{L^3}$$

In which CF = conditioning factor, W = wet weight (g), L = length (cm).

### 2.2.2 Specific growth rate

Specific growth rate (SGR) was calculated according to the following formula:

$$SGR = \frac{(lnW_2 - lnW_1)}{(t_2 - t_1)} * 100$$

In which $t_1$ = start point of trial, $t_2$ = endpoint of trial, $\ln W_1$ = natural logarithm of weight (g) at $t_1$, $W_2$ = natural logarithm of weight (g) at $t_2$.

### 2.2.3 Urine flow rate (UFR)

Urine flow rate was calculated in accordance with Curtis and Wood (1991) using this formula:

$$UFR = \frac{\sum V_{total}}{mass * time}$$

In which UFR = Urine Flow Rate, $\sum V_{total}$ = total volume of collected urine (ml), mass = fish wet weight (g), and time = time passed (hours).

### 2.2.4 Urine and plasma bicarbonate ($HCO_3^-$) and partial pressure $CO_2$ ($PCO_2$)

Bicarbonate ($HCO_3$-) and $PCO_2$ in both urine and plasma was calculated using the Henderson-Hasselbalch equation:

$$pH_s = pK_{app} + \log_{10}\frac{[HCO_3^-]}{[CO_2]} = pK_{app} + \log_{10}\frac{[HCO_3^-]}{PCO_2 * \alpha CO_2}$$

In which $pH_s$ = pH for each sample, $pK_{app}$ = apparent dissociation constant for $CO_2$ hydration, $[CO_2]$ = dissolved carbon dioxide (mM), $[HCO_3$-$]$ = dissolved bicarbonate (mM), $\alpha CO_2$ = solubility coefficient of $CO_2$, and $PCO_2$ = partial pressure of $CO_2$.

$\alpha CO_2$ and $pK_{app}$ was calculated in accordance with (Boutilier *et al.*, 1985) using the following formulas and are valid only for the temperature range 5-15°C:

$$\alpha CO_2 = 1.0064 * 10^{-1} - 5.4431 * 10^{-3}(T) + 2.1776 * 10^{-4}(T^2) - 4.9731 * 10^{-6}(T^3) + 4.5288 * 10^{-8}(T^4), mmol * L^{-1} * Torr^{-1}$$

In which T = temperature in °C.

$$pK_{app} = 6.4755 * T^{-0.0187} + \log_{10} T\,(1.1704 - 0.1672) + 0.1073 * pH_s - 0.7511$$

In which T = temperature in °C and $pH_s$ = sample pH.

Bicarbonate and $PCO_2$ were calculated from measured total $CO_2$ ($TCO_2$), extracellular pH and temperature, and by implementing the plasma solubility coefficient of $CO_2$ ($\alpha CO2$ = 0.063 mmol/L/mmHg) and the apparent dissociation constant for $CO_2$ hydration ($pK_{app}$ = 6.86 – 0.087*pH) for trout at 14°C (Boutilier, Heming and Iwama, 1984; Boutilier *et al.*, 1985).

### 2.2.5 Urine-Plasma ratio

Urine plasma ratios were calculated using the following equation:

$$UP\ ratio = \frac{[U]}{[P]}$$

Where [U] = solute concentration in urine and [P] = solute concentration in plasma.

### 2.2.6 Urine-Plasma fractional excretion rate (%)

Urine-Plasma fractional excretion rate was calculated in accordance with Brown *et al.* (2015) using the following formula based on spot urine and serum samples:

$$ER_x = \frac{U_x P_{cr}}{U_{cr} P_x}$$

In which $U_x$ = urine concentration of solute, $U_{cr}$ = urine concentration of creatinine, $P_x$ = plasma concentration of solute and $P_{cr}$ = plasma concentration of creatinine.

## 2.3 Lab analysis

### 2.3.1 $Na^+/K^+$ ATPase enzyme activity analysis

The NKA enzyme activity was measured for gills, kidney and intestine using a Tecan Sunrise microplate reader (Tecan, Mannedorf, Switzerland) using a micro assay method described by (McCormick, 1993) that is further modified for analysis of intestine (Sundell and Sundh, 2012) and kidney (Takvam *et al.*, 2024) tissues.

For the intestinal NKA activity, it's important to run the analysis of the samples within 3 days after sampling, preferably the following day within 24 hours, to limit loss of NKA enzyme activity due to the digestive properties of the intestine and the potential for rapid degradation (Takvam *et al.*, 2024).

Before analysis, an assay mixture (AM: 5,0 U/ml Pyruvate Kinase (PK), 4,0 U/ml Lactic Dehydrogenase (LDH), 21 mM phosphenylpyruvate (PEP), 0,22 mM beta-Nicotinamide Adenine Dinuclotide (NADH), 0,7 mM Adenosine Triphosphate (ATP) and 50 mM imidazole (IB)) was prepared. This served as the basis for the two working solutions, one without (AM) and one with (AM-O) 0,5 mM of the ATPase enzyme inhibitor Oubain. Finally, a salt solution (50 mM Imidazole (IB), 189 mM NaCl, 10,5 mM $MgCl_2$, 42 mM KCl) was added to both working solutions to complete the AM and AM-O mixtures. Before any analysis, the assay mixture was tested by running an ADP standard curve in quadruplicates of 10 µl, at four different concentrations. The assay mixture is prepared correctly and in good condition if the negative slope of the endpoint standard curve is between 17-19 mOD/nmole ADP.

Samples were thawed and kept on ice during the analysis up until adding the assay mixture into each microplate well. Gill filaments (n = 2-4) and kidney tissue (1.2-1.6 mg) were submerged into Eppendorf vials (0,6 ml) containing 100 µl SEI buffer. Intestinal samples are very sensitive to degradation and 1-1.4 mg of intestinal tissue was already in Eppendorf vials (0.6 ml) from sampling (see above). Then, 25 µl of SEID (20ml SEI buffer, 0.1g Na-deoxycholate) were added and tissue samples homogenized with a motor pestle (VWR 431-0100) for 20 seconds. The homogenized samples were then centrifuged (6500 RCF for kidney and gills/8000 RCF for intestine, 4 minutes, 4°C) to precipitate cell debris. For each sample, quadruplets of 10 µl of the supernatant were pipetted into their designated wells in a 96-well Nunc microplate, while 10 µl triplicates were pipetted into a 96-well Costar microplate (CLS9017, Sigma) for protein analysis. The working solutions, AM and AM-O, were heated to 25°C in a water bath before 200 µl of each of the working solutions were added to half of the four replicates on the Nunc

microplate. After the working solution was added, the plate was quickly inserted into the plate reader, and absorbance was measured at a wavelength of 340 nm at 25°C with 60 cycles at 10 second intervals for a total running time of 10 minutes. The NKA activity in the tissues are enzymatically coupled with pyruvate kinase and lactic dehydrogenase and causes oxidation of NADH resulting in an equimolar disappearance of NADH which is outputted by the machine as mOD (milli optical density unit)/10µl/minute. The mOD/10µl/minute is divided by the standard curve slope (mOD/nmole ADP) to convert the measurement to nmoles ADP/10µl/minute using the following formula:

$$\frac{Na^+/K^+ - ATPase\ (mOD/10\mu l/minute)}{ADP\ standard\ curve\ (mOD/nmole\ ADP)} = nmoles\ ADP/10\mu l/minute$$

Concentration of protein in the samples were measured by using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Massachusetts; USA). A protein working solution was a made up from the BCA Protein Reagents (50 parts A: 1 part B), while standard solutions at four different concentrations (0, 5, 10, 20 µl BSA standard mixture / 10 µl of deionized water ($DiH_2O$)) were made up from BSA standard mixture. Triplicates of 10 µl for each standard were pipetted into the empty wells on the Costar microplate, and then 200 µl of the protein working solution was added to each triplicate sample and standard. The plate was then covered with parafilm and gently shaken for 30 seconds, before being incubated at 37°C for 30 minutes. The plate was cooled for 2-3 minutes after incubation, before being inserted in the plate reader and absorbance was measured at a wavelength of 562nm.

After analyzing the NKA activity and protein concentration in the samples, we could then calculate the ATPase activity by dividing the nmoles ADP/10µl/minute by the protein concentration, and then multiply by 60, to get the NKA enzyme activity in µmoles ADP per mg protein per hour, using these formulas:

$$\frac{nmoles\ ADP/10\mu l/min}{\mu g/10\mu l protein} = \mu molesADP/mg\ protein/minute$$

$$(\mu molesADP/mg\ protein/minute)(60\ minutes) = \mu molesADP/mg\ protein/hour$$

### 2.3.2 Hematological analysis

Hematocrit (Hct) was quantified as the fractional volume of packed RBCs after centrifugation (3 minutes, 12000 g). Hemoglobin was determined using the haemoglobincyanide method

(HiCN) described by Drabkin and Austin (1935), with slight modifications for cold water fish as described by Blaxhall and Daisley (1973). Briefly, 10 µl blood were added to 1 ml of Drabkin's solutions, thoroughly vortexed and 200 µl pipetted into a microwell plate and measured at 540 nm in a Tecan Sunrise plate reader. Mean corpuscular hemoglobin concentrations (MCHC) was calculated as the ratio of Hb to Hct.

### 2.3.3 Plasma and urine ion and metabolite analysis

Ion and metabolite composition analysis for urine and plasma samples was measured using a Pentra C400 Clinical Chemistry Analyzer (HORIBA, Kyoto Prefecture, Japan) with an ISE (Ion Selective Electrode) module and following the manufacturer protocol. All reagents were calibrated using ABX Pentra Multical, and calibration was approved using the ABX Pentra N and P controls. The ISE module was calibrated using the ABX Pentra Standard 1, ABX Pentra Standard 2 and ABX Pentra Reference. All of the ABX Pentra solutions were supplied by HORIBA. It is important to mention that this machine is intended for human plasma and serum, and the reference ranges according to the manufacturer protocol differ from the ranges in our samples.

Urine and plasma samples were thawed and kept on ice during the analysis up until inserting the samples in the Pentra machine. Thawed samples were vortexed using a vortex mixer and centrifuged using VWR Mini Star Whiteline Microcentrifuge (Prod. number 21110362).

Due to low urine volumes, ranging from 25-150 µl, urine samples were diluted with MilliQ water for enough volume to run all analysis on the Pentra machine. Urine sample volumes over 35 µl was diluted 1/3, while lower volumes were diluted ¼. Further dilutions were undesirable as they are prone to inaccurate readings due to ion and metabolite concentrations being too low. Urine volume up to 100 µl was pipetted into Pentra specific aliquots and diluted with Milliq water, before being vortexed and centrifuged again before analysis. After analysis, the ion concentrations measured were multiplied by 3 or 4 respectively to get the concentration of undiluted urine. The parameters measured for urine were as follows: total $CO_2$, calcium, magnesium, phosphorus, albumin, creatinine, total protein, sodium, chloride and potassium.

For plasma samples, a dilution was not necessary, and 400 µl was pipetted into Pentra specific aliquots. The plasma samples were vortexed and centrifuged before analysis. The parameters measured for plasma samples were the same as for urine, but for glucose and lactic acid were also measured.

*Calcium:*

Calcium concentration was measured according to the method based on metallochromogen Arsenazo III, using the ABX Pentra Calcium AS CP reagent (HORIBA). At pH 6.75, calcium ions ($Ca^{2+}$) react with Arsenazo III (2,2'-[1,8-Dihydroxy-3,6-disulphonapthyylene-2,7-bisarzo]-bisbenzenearsonic acid), creating a Ca-arsenazo III complex with an intense purple color. The absorbance of the Ca-arsenazo III complex was measured bichromaticcally at 660/700 nm (Michaylova and Ilkova, 1971). The calcium concentration in the samples was directly proportional to the increase in absorbance of the reaction mixture. Arsenazo III has a high affinity for calcium ions ($K°=1x10^{-7}$), and according to the manufacturer protocol, it shows no interference from other cations typically present in urine and plasma.

$$Ca^{2+} + Arsenazo\ III \overset{pH\ 6.75}{\longleftrightarrow} Ca - Arsenazo\ III\ complex\ (purple)$$

*Chloride, potassium and sodium:*

Chloride, potassium and sodium concentrations were measured by potentiometry, using the ISE (Ion Selective Electrode) module. Each sample was measured with parallel electrodes, one for each of the ions, with special membranes and solutions with a known concentration of the different ions. The ions in the samples created a difference in potential over the electrode membranes, which was compared to the reference electrode (Buck, 1981) according to the manufacturing protocol.

*Magnesium:*

Magnesium was measured using a method based on a photometric test using xylidl blue. The ABX Pentra Magnesium RTU reagent (HORIBA) is an alkaline solution containing both xylidl blue and GEDTA (glycoletherdiamine-tetraacetic acid). In the alkaline solution, magnesium ions ($Mg^{2+}$) reacted with xylidl blue and formed a purple-colored complex. The GEDTA complexes the calcium ions ($Ca^{2+}$) present and makes the magnesium-xylidl blue reaction specific. The absorption of the magnesium-xylidl blue complex was measured at 520/700nm in a photometric test (Burcar, Boyle and Mosher, 1964), where the intensity of the purple color was proportional to the magnesium concentration according to the manufacturer protocol.

*Phosphorous:*

Phosphorous concentration was measured based on a UV method using phosphomolybdate. Using the ABX Pentra Phosphorous CP reagent, it created an acidic medium with ammonium molybdate, where phosphate reacted to form a yellow colored phosphomolybdate complex measured at 340 nm (Daly and Ertingshausen, 1972). The concentration of inorganic phosphorous is proportional to the intensity of the phosphomolybdate complex coloration.

$$Ammonium\ molybdate + Sulphuric\ acid \xrightarrow{Phosphorou} Phosphomolybdate\ complex\ (yellow)$$

*Total protein:*

Total protein was measured by using ABX Pentra Total Protein CP reagent in a colorimetric test. This method was based the Biuret reaction (Gornall, Bardawill and David, 1949), were the formation of a purple colored complex between Biuret ($HN(CONH_2)2$) and two consecutive peptide connections, in and alkaline solution and the presence of copper ions. The intensity of the coloration is directly proportional to the protein concentration.

$$Proteins + Cu^{2+} \rightarrow Coloured\ complex$$

*Creatinine:*

Creatinine was measured by using ABX Pentra Creatinine 120 CP reagent, and method based on the reaction between creatinine and sodium picrate, in an alkaline solution, to form Janovsky complex (Jaffe, 1886). The increase in absorbance for the Janovsky complex is measured at 510 nm and is directly proportional to the creatinine concentration.

$$Creatinine + Alkaline\ picrate \rightarrow creatinine - picrate\ complex$$

*Albumin:*

Albumin was measured using the ABX Pentra Albumin CP reagent in a colorimetric test, where Bromocresol Green (BCG) selectively reacted to albumin forming a blue-colored complex. The reaction requires a succinate buffer and a nonionic surfactant Brij35, at pH 4.2, and is measured at 628 nm, where the intensity of the coloration of the complex is directly proportional to the concentration of albumin (Doumas, Ard Watson and Biggs, 1971).

$$Albumin + BCG \xrightarrow{pH\ 4.20} Albumin - BCG\ complex$$

*Total $CO_2$:*

Measurements of $CO_2$ were based on an enzymatic test with the ABX Pentra CO2 RTU reagent containing phosphoenolpyruvate (PEP), phosphoenolpyruvate carboxylase (PEPC) and a stable analog for nicotinamide adenine dinucleotide (NADH). PEP reacted with bicarbonate ($HCO_3$-), using PEPC and magnesium ions, to form oxaloacetate and dihydrogen phosphate ($H_2PO_4$-).

$$Phosphoenolpyruvate + HCO_3^- \xrightarrow{PEPC+Mg^{2+}} Oxaloacetate + H_2PO_4^-$$

This removed bicarbonate and disturbed the following equilibrium:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

$CO_2$ was then converted to bicarbonate and was included in the first reaction. A second reaction is with the formed oxaloacetate and a reduced cofactor, using malate hydrogenase (MDH), to form malate and a cofactor.

$$Oxaloacetate + Reduced\ cofactor \xrightarrow{MDH} Malate + Cofactor$$

The concentration of total $CO_2$ in the samples is then proportional to the decrease of reduced cofactor concentration measured at 405 nm (Norris, Atkinson and Smith, 1975).

*Glucose:*

Glucose in plasma was measured using the ABX Pentra Glucose HK CP reagent and an enzymatic method in a colorimetric test. With hexokinase present, Glucose was phosphorylated by ATP and formed glucose-6-phosphate, which was oxidized by glucose-6-phosphate dehydrogenase (G6P-DH) when $NAD^+$ was present and formed D-glucose-6-phosphate and NADH. The reduction of $NAD^+$ and the production of NADH is directly proportional to the concentration of glucose and absorbance is measured at 340 nm (Kubihal *et al.*, 2021).

$$D - glucose + ATP \xrightarrow{Hexokinase} Glucose - 6 - phosphate + ADP$$

$$Glucose - 6 - phosphate + NAD^+ \xrightarrow{G6P-DH} D - glucose - 6 - phosphate + NADH$$

*Lactic acid:*

Lactic acid in plasma was measured using ABX Pentra Lactic Acid and the Trinder method, an enzymatic colorimetric method (Trinder, 1969). Lactate oxidase triggered the release of hydrogen peroxide ($H_2O_2$) from lactic acid and $O_2$. With peroxidase present, hydrogen peroxide then reacted with 4-aminoantipyrine (4AAP) and N-Ethyl-N-(3-sulfopropyl)-m-anisidine (ADPS) and formed a colored Quinoneimine complex. The concentration of lactic acid in the sample was proportional to the intensity of the complex color.

$$Lactate + O_2 \xrightarrow{Lactate\ oxidase} Pyruvate + H_2O_2$$

$$2H_2O_2 + 4AAP + ADPS \xrightarrow{Peroxidase} Quinoneimine\ complex + 4H_2O$$

## 2.4 Statistical analysis

All statistical analysis was performed using Prism (GraphPad Prism version 10.4.1). All the collected data was formatted in Excel (version 2502) before being imported to Prism for analysis and plotting. Normality for all data was evaluated using a Shapiro-Wilk test. A natural logarithmic transformation for normalization was performed for any data not normally distributed prior to analysis. For all parameters, a two-way ANOVA was run for $CO_2$ exposure and feed formulations, while a one-way ANOVA was run for comparing sampling times within each group. These analyses were followed by Tuckey's post hoc multiple comparisons test to do pair-wise comparisons for any significant differences between sampling times, $CO_2$ exposure and feed formulations. For the one-way ANOVA, if any data was not normally distributed after natural logarithmic transformation, a non-parametric Kruskal-Wallis test was run instead. Significant difference was determined by a p-value of less than 5% ($p<0.05$). All data was presented as standard error of the mean (SEM).

# 3. Results

As described in experimental design, groups were run at control or experimental $CO_2$ treatment with low salt (A), medium salt (B) or high salt (C) diets. Each of these groups are referred to by group names indicating $CO_2$ treatment and assigned salt diets. Control $CO_2$ treatment groups are therefore named Control-A, Control-B and Control-C, while experimental $CO_2$ groups are named High-A, High-B and High-C.

## 3.1 $CO_2$ drop trial

### 3.1.1 Animal performance

*Body mass (g)*

For each sampling time, the mean body mass of fish in the control groups was slightly but not significantly higher than fish in experimental groups, while specific salt diets did not affect body mass between groups (Fig. 5a).

For control groups, mean body mass only changed significantly for Control-C increasing from 289.2±15.2 g pre-drop and 316.8±13.3 ($p<0.0203$) 5hr post-drop, to 374.8±14.7 g ($p<0.0006$) 2W post-drop (Appendix A. table A.3). Body mass had increased significantly for experimental groups High-A and High-B at the 2W post-drop sampling, but less than control groups, with High-A increasing from 244±13.4 g pre-drop to 313.17.7 g ($p<0.0139$) at 2W post-drop, while High-C increased from 267± 16.0 g at the 5hr post-drop to 321.13.3 ($p<0.0415$) at the 2W post-drop (Appendix A. table A.3).

*Specific growth rate (SGR) %*

Mean specific growth rate (SGR) was generally higher for control groups compared to experimental groups within each sampling time, albeit not significant (Fig 5b). Additionally, no significant changes were observed between sampling times for within groups (Appendix A, table A.3).

*Condition factor (CF)*

The mean condition factor did not differ significantly between groups at each sampling time (Fig. 5c). For both control and experimental groups, mean condition factor for medium and high salt diets had a significant reduction from pre-drop to 2W post-drop, with Control-B having decreased to 1.08±0.06 at 2W post-drop from 1.36±0.10 ($p<0.0116$) at pre-drop, while Control-C had decreased to 1.13±0.02 at 2W post-drop from 1.25±0.02 ($p<0.0010$) at pre-drop and 1.22±0.02 ($p<0.0073$) at 5hr post-drop (Appendix A, table A.3). The experimental group High-

B had a mean condition factor of 1.28±0.05 pre-drop that decreased to 1.09±0.03 ($p<0.0027$) at 2W post-drop, while High-C was 1.27±0.04 pre-drop and decreased to 1.15±0.02 ($p<0.0375$) at 2W post-drop (Appendix A, table A.3).

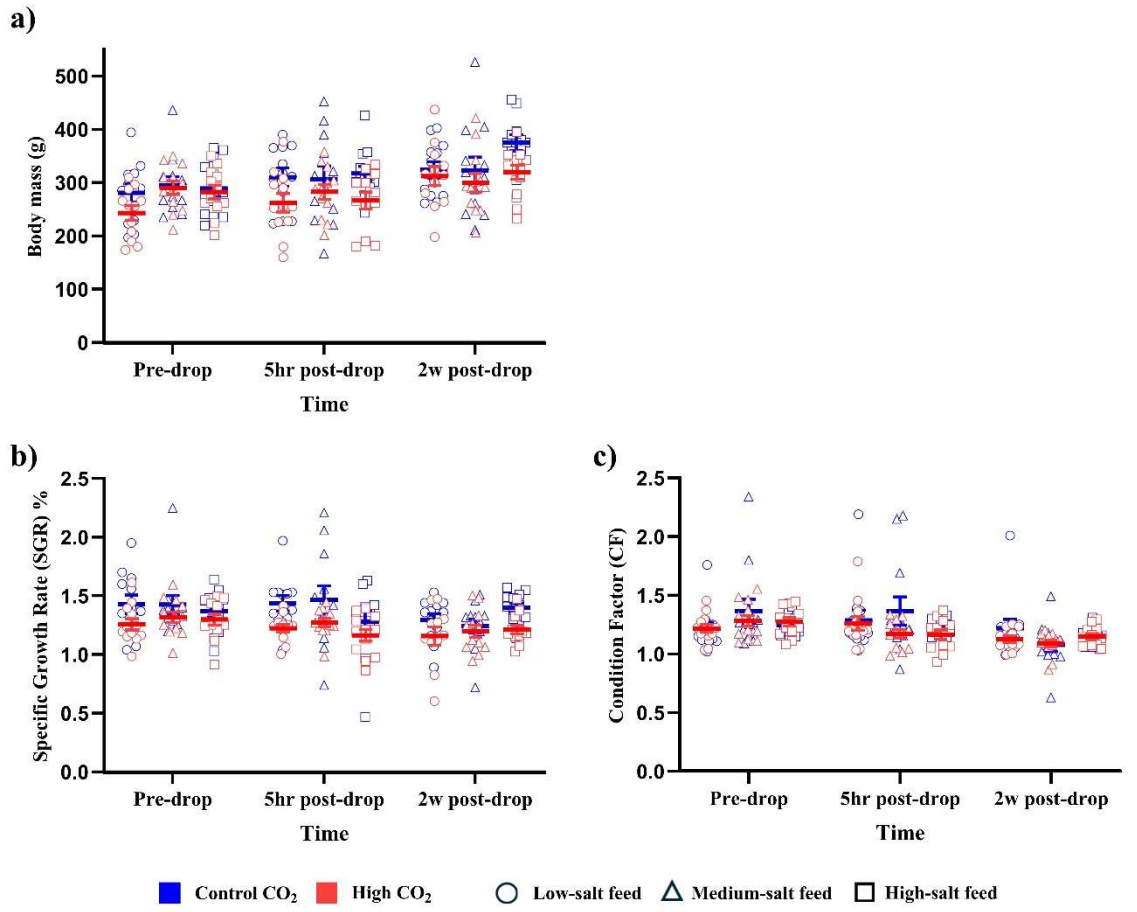


**Figure 5: Body mass (g), Specific Growth Rate (%) and Condition Factor of juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

## 3.1.2 Acid-base regulation

### *Blood pH*

Mean blood pH for experimental groups was higher than control groups for the pre-drop and 5hr post-drop sampling, while control had higher mean blood pH than experimental groups at the 2W post-drop sampling (Fig. 6a). However, the only significant difference was for low salt diet groups with High-A at 7.5±0.04 being higher than Control-A at 7.3±0.03 ($p<0.0070$, Fig. 6a).

All control groups had a significant increase in mean blood pH at 2W post-drop, with Control-A at 7.5±0.04 having increased from pre-drop ($p<0.0004$) and 7.3±0.05 ($p<0.0002$) 5hr post-drop, Control-B at 7.6±0.05 increased from 7.3±0.03 ($p<0.0001$) pre-drop and 7.4±0.04 ($p<0.0003$) 5hr post-drop, and Control-C at 7.5±0.03 rising from 7.3±0.03 ($p<0.0016$) pre-drop and 7.4±0.04 ($p<0.0191$) 5hr post-drop (Appendix A, table A.4). No significant differences were found between sampling times for the experimental group (Appendix A, table A.4).

### *Plasma bicarbonate [$HCO_3^-$] (mM)*

The mean plasma bicarbonate concentration was significantly higher for experimental groups compared to their respective control groups at pre-drop, where High-A was 22.7±0.9 mM compared to 14.6±0.9 mM ($p<0.0001$) for Control-A, High-B was 22.5±0.8 mM compared to 19.2±1.3 mM ($p<0.0386$) for Control-B, and High-C was 24.5±0.9 mM compared to Control-C at 15.0±0.5 mM ($p<0.0001$) (Fig. 6b). Additionally, the medium salt diet group Control-B had a significantly higher bicarbonate concentration compared to the low salt diet group Control-A ($p<0.0011$) and the high salt diet group Control-C ($p<0.0034$) (Fig. 6b). The plasma bicarbonate concentration for experimental groups were still higher than control groups for the 5hr post-drop sampling, but only low and medium salt diets were significantly higher, with High-A being 18.8±0.8 mM compared to Control-A at 13.5±0.08 mM ($p<0.0001$), and High-B was 17.9±0.5 mM compared to Control-C at 13.0±0.7 mM ($p<0.0004$) (Fig. 6b). At 2W post-drop control groups with medium and high salt diet had a significantly higher plasma bicarbonate concentration than their respective experimental groups, with Control-B having 16.0±1.1 mM compared to High-B at 12.4±0.5 mM ($p<0.0168$), and Control-C being 15.9±0.9 compared to High-C at 12.2±0.6 mM ($p<0.0177$) (Fig. 6b). Within control groups, the low salt diet group Control-A was 10.9±0.3 mM at 2W post-drop and were significantly lower than Control-B ($p<0.0002$) and Control-C ($p<0.0003$) (Fig. 6b).

Within control groups, the mean plasma bicarbonate concentration for the low salt diet group Control-A decreased significantly from pre-drop to 10.9±0.3 mM ($p<0.0036$) at 2W post-drop, the medium salt diet group Control-B had a decrease from pre-drop to 5hr post-drop ($p<0.0008$), while the high salt diet group Control-C decreased from pre-drop to 5hr post-drop ($p<0.0274$) and then increased to 2W post-drop ($p<0.0025$) (Appendix A, table A.4). For all the experimental groups, bicarbonate concentration decreased significantly from pre-drop to 5hr post-drop and then decreased further for the 2W post-drop sampling. The low salt diet group High-A started at 22.7±0.9 mM pre-drop, then decreased to 18.8±0.8 mM ($p<0.0063$) at 5hr post-drop, ending at 12.2±0.6 mM (Pre-drop: $p<0.0001$, 5hr: $p<0.0014$) (Appendix A, table

A.4). The medium salt diet group High-B was 22.5±0.8 mM at pre-drop, then lowered to 17.9±0.5 (P<0.0001) for the 5hr post-drop, and decreased further to 12.4±0.5 mM (Pre-drop: p<0.0001, 5hr: p<0.0001). Finally, the high salt diet group High-C started at 24.5±0.9 mM pre-drop, decreased to 16.4±0.9 mM (p<0.0001) for the 5hr post-drop, and then decreased to 12.2±0.6 (Pre-drop: p<0.0001, 5hr: p<0.0014) for the 2W post-drop (Appendix A, table A.4).

### *Plasma $P_aCO_2$ (mmHg)*

Generally, there were no significant difference for mean plasma partial pressure $CO_2$ between control and experimental groups, except for Control-C at 15.2±0.9 mmHg that were significantly lower than High-C at 20.724±2.5 mmHg (p<0.0129) pre-drop. Additionally, at 5hr post-drop the experimental groups High-A at 16.9±1.1 mmHg had significantly higher plasma partial pressure $CO_2$ than High-C at 11.0±0.8 mmHg (p<0.0060, Fig. 6c).

The mean plasma partial pressure $CO_2$ had decreased at 2W post-drop for low salt diet groups with Control-A ending at 7.4±0.7 mmHg compared to 16.7±1.2 mmHg (p<0.0001) pre-drop and 15.7±1.0 mmHg (P<0.0001) 5hr post-drop, while High-A ended at 8.7±0.7 mmHg compared to 17.3±1.9 mmHg (P<0.0002) pre-drop and 16.9±1.1 mmHg (p<0.0003) 5hr post-drop (Appendix A, table A.4). The control group with medium salt diet Control-B was 17.0±1.0 mmHg pre-drop and significantly decreased to 13.0±1.1 mmHg (p<0.0133) for 5hr post-drop and ended at 8.9±0.7 mmHg (pre-drop: p<0.0001, 5hr: p<0.0121) for 2W post-drop (Appendix A, table A.4). The mean partial pressure $CO_2$ of Control-C decreased significantly from pre-drop to 10.6±0.6 mmHg (p<0.0066, Appendix A, table A.4) at 2W post-drop.

The experimental group with medium salt diet High-B had a mean plasma partial pressure $CO_2$ of 19.0±1.6 mmHg pre-drop and significantly decreased to 14.7±0.9 mmHg (p<0.0308) 5hr post drop and significantly lowered to 9.1±0.8 mmHg (Pre-drop: p<0.0001, 5hr: p<0.0036) (Appendix A, table A.4). Finally, the experimental high salt diet group High-C was significantly higher pre-drop at 20.7±2.5 compared to 11.0±0.7 (P<0.0002) 5hr post-drop and 11.0±0.8 (P<0.0002) 2W post-drop (Appendix A, table A.4).

### *Plasma Total $CO_2$ (mM)*

The mean plasma total $CO_2$ concentrations for experimental groups were significantly higher than their respective control groups for the pre-drop sampling, with High-A at 23.8±0.9 mM compared to 15.7±1.0 mM (p<0.0001) for Control-A, High-B at 23.7±0.8 mM compared to 17.7± 0.8 mM (p<0.0001) for Control-B, and High-C at 25.8±0.8 compared to 15.9±0.6 (p<0.0001) (Fig. 6d). The mean plasma $CO_2$ concentration for the experimental groups was still

higher than control groups for the 5hr post-drop sampling, with High-A being 19.9±0.9 mM compared to 14.5±0.8 mM ($p<0.0001$) for Control-A, High-B was 18.8±0.5 mM compared to 13.9± 0.7 mM ($p<0.0001$) for Control-B, while High-C was 17.1±0.8 mM compared to 13.1±0.5 mM ($p<0.0037$) for Control-C (Fig. 6d). For the 2W post-drop sampling, the mean plasma total $CO_2$ was higher for control groups with medium and high salt diets compared to their respective experimental groups, where Control-B was 16.6±1.1 mM compared to 12.9±0.5 mM ($p<0.0122$) for High-B, and Control-C was 16.5±1.0 mM compared to 12.9±0.6 mM ($p<0.0135$) for High-C (Fig. 6d). Additionally, the control groups with low salt diet had significantly lower mean plasma total $CO_2$ concentration with Control-A being 11.4±0.3 compared to Control-B ($P<0.0001$) and Control-C ($P<0.0001$) (Fig. 6d).

Within control groups, the mean plasma total $CO_2$ concentration for the low salt diet group Control-A had significantly decreased for the 2W post-drop sampling compared to pre-drop ($p<0.0010$) and 5hr post-drop ($p<0.0180$), the medium salt diet group Control-B decreased significantly from pre-drop to 5hr post-drop ($p<<0.0119$), and the high salt diet group Control-C were significantly lower for 5hr post-drop compared to pre-drop ($p<0.0204$) and 2W post-drop ($p<0.0042$) (Appendix A, table A.4). The mean plasma total $CO_2$ concentration decreased significantly for all experimental groups, with the low salt diet group High-A decreasing from pre-drop to 5hr post-drop ($p<0.0087$) and then decreasing to 12.9±0.5 mM (Pre-drop: $p<0.0001$, 5hr: $p<0.0001$) at 2W post-drop, the medium salt diet group decreased from pre-drop to 5hr post-drop ($p<0.0001$) and then to the 2W post-drop (Pre-drop: $p<0.0001$, 5hr: $p<0.0001$), while the high salt diet group High-C also decreased from pre-drop to 5hr post-drop ($p<0.0001$) and then to the 2W post-drop (Pre-drop: $p<0.0001$, 5hr: $p<0.0001$) (Appendix A, table A.4).

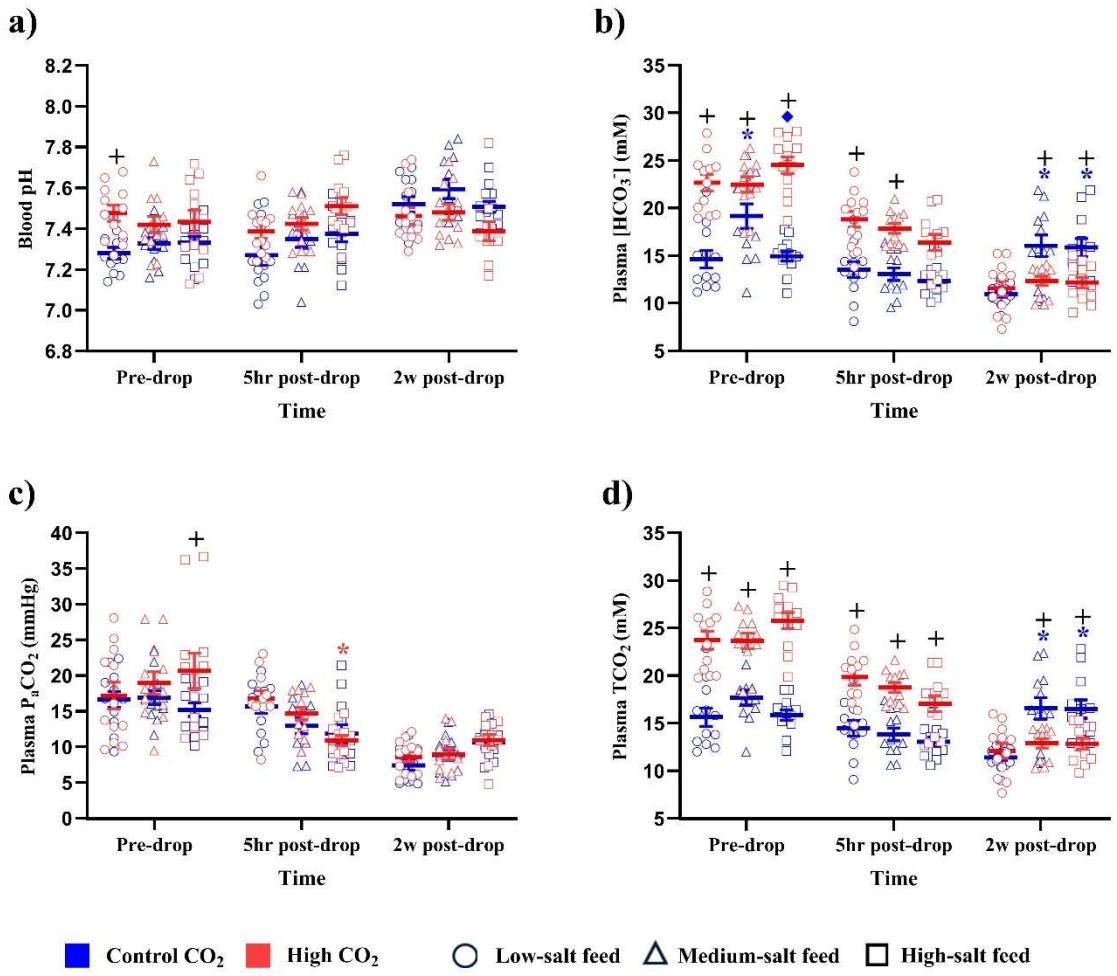


**Figure 6: Plasma acid-base parameters of juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Within the control $CO_2$ groups, blue asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while blue diamond (◆) indicates a significant difference between medium and high salt diet. within the control $CO_2$ group. Within high $CO_2$ groups, red asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet. Pluss sign (+) indicates a significant difference between control and high $CO_2$ treatment within diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

### *Urine pH*

The experimental groups did generally have a higher Urine pH than control groups, but the only significant difference was between medium salt diet groups at the pre-drop sampling, with High-B at 7.9±0.03 being significantly higher than Control-B at 7.4±0.09 ($p<0.0067$, Fig. 7a). Between the experimental groups at pre-drop, the urine pH was significantly lower for the low salt diet group High-A compared to both the medium salt diet group High-B at 7.9±0.03 ($p<0.0093$) and the high salt diet group High-C at 7.9±0.05 ($p<0.0102$) (Fig. 7a).

Between sampling times, only the experimental groups with medium salt diet changed significantly, with High-B having decreased to 7.5±0.10 at 2W post-drop from both pre-drop ($p<0.0047$) and 7.8±0.06 ($p<0.0352$) at the 5hr post-drop (Appendix A, table A.5).

### *Urine bicarbonate [$HCO_3^-$] (mM)*

Mean urine bicarbonate concentrations were significantly higher for experimental groups compared to respective control groups for the pre-drop sampling, with High-A at 6.7±1.4 mM compared to Control-A at 1.4±0.4 mM ($p<0.0001$), High-B was 8.2±1.0 mM compared to Control-B at 2.5±0.4 mM ($P<0.0001$), and High-C was 8.9±1.1 mM compared to Control-C at 3.6±0.8 mM ($P<0.0001$) (Fig. 7b). For 5hr post-drop only High-B at 5.5±0.9 mM was significantly higher than Control-B at 2.1±0.6 mM ($p<0.0368$, Fig. 7b). Additionally for experimental groups, the low salt diet group High-A at 1.7±0.4 mM had significantly lower bicarbonate concentrations compared to both the medium salt diet group High-B ($p<0.0136$) and the high salt diet group High-C at 5.0±1.7 mM ($p<0.0392$) for 5hr post-drop (Fig. 7b). At 2W post-drop, the low salt diet was significantly higher than the high salt diet for experimental groups, with High-A at 4.2±0.8 mM compared to High-C at 0.5±0.1 mM ($p<0.0128$, Fig. 7b).

The control groups with low and high salt diets had a slight but significant decrease in mean urine bicarbonate concentrations, with Control-A being 3.3±0.6 mM for 2W post-drop having decreased from pre-drop ($p<0.0079$) and 1.4±0.3 mM ($p<0.0111$) from 5hr post-drop, while Control-C was 0.6±0.1 mM for 2W post-drop having decreased from pre-drop ($p<0.0103$) and 4.4±0.7 mM ($p<0.0002$) at 5hr post-drop (Appendix A, table A.5). All experimental groups had a significant decrease in urine bicarbonate, with the low salt diet group High-A decreasing from pre-drop to 5hr post-drop ($p<0.0021$), the medium salt diet group High-B decreased significantly from both pre-drop and 5hr post-drop to 2.0±0.5 mM (Pre-drop: $p<0.0001$, 5hr: $p<0.0004$) at 2W post-drop, while the high salt diet group High-C decreased from pre-drop to 5hr post-drop ($p<0.0226$) and then further to 2W post-drop (Pre-drop: $p<0.0001$, 5hr: $p<0.0001$) (Appendix A, table A.5).

### *Urine $PCO_2$ (mmHg)*

Mean urine partial pressure $CO_2$ was only significantly different between experimental and control groups at pre-drop between low salt diets, with High-A at 5.8±1.8 mmHg being higher than Control-A at 1.2±0.2 mmHg ($p<0.0001$, Fig. 7c). Additionally, High-A had significantly higher urine partial pressure $CO_2$ than both the medium salt diet group High-B at 2.4±0.3 mmHg ($p<0.0001$) and the high salt diet group at 2.5±0.3 mmHg ($p<0.0001$) (Fig. 7c).

The low salt diet group was the only control group that changed significantly between sampling times, with Control-A going from pre-drop and 0.9±0.2 mmHg at 5hr post-drop to 2.3±0.3 mmHg (Pre-drop: $p<0.0082$, 5hr: $p<0.0009$) at 2W post-drop (Appendix A, table A.5). For experimental groups, urine partial pressure $CO_2$ decreased, with the low salt diet group High-A decreasing significantly from pre-drop to 0.9±0.2 mmHg ($p<0.0001$) 5hr post-drop and 1.4±0.4 mmHg ($p<0.0006$) 2W post-drop, the medium salt diet group High-B had a significant decrease from pre-drop to 1.1±0.2 mmHg ($p<0.0006$) 2W post-drop, and the high salt diet group High-C decreased from pre-drop to 1.3±0.2 ($p<0.0311$) 5hr post-drop and then further to 0.2±0.1 mmHg (Pre-drop: $p<0.0001$, 5hr: $p<0.0001$) for the 2W post-drop (Appendix A, table A.5).

### *Urine Total $CO_2$ (mM)*

Mean urine total $CO_2$ concentrations were significantly higher for experimental groups compared to respective control groups for the pre-drop sampling, with High-A at 7.0±1.4 mM compared to Control-A at 1.5±0.4 mM ($p<0.0001$), High-B was 8.4±1.0 mM compared to Control-B at 2.6±0.4 mM ($p<0.0001$) and High-C was 9.1±1.1 mM compared to Control-C at 3.7±0.8 mM ($p<0.0001$) (Fig. 7d). For 5hr post-drop only High-B at 5.6±0.9 mM was significantly higher than Control-B at 2.2±0.6 mM ($p<0.0349$, Fig. 7d). Additionally for experimental groups, the low salt diet group High-A at 1.7±0.4 mM had significantly lower urine total $CO_2$ concentration compared to both the medium salt diet group High-B at 5.6±0.9 mM ($p<0.0125$) and the high salt diet group High-C at 5.1±1.7 mM ($p<0.0378$) for 5hr post-drop (Fig. 7b). At 2W post-drop, the low salt diet was significantly higher than the high salt diet for experimental groups, with High-A at 4.3±0.8 mM compared to High-C at 0.5±0.1 mM ($p<0.0107$, Fig. 7d).

The control groups with low and high salt diets had a slight but significant decrease in mean urine total $CO_2$ concentrations, with Control-A being 3.5±0.6 mM for 2W post-drop having decreased from pre-drop ($p<0.0058$) and 1.4±0.3 mM ($p<0.0038$) from 5hr post-drop, while Control-C was 0.6±0.1 mM for 2W post-drop having decreased from pre-drop ($p<0.0079$) and 2.2±0.6 mM ($p<0.0002$) at 5hr post-drop (Appendix A, table A.5). All experimental groups had a significant decrease in urine bicarbonate, with the low salt diet group High-A decreasing from pre-drop to 5hr post-drop ($p<0.0010$), the medium salt diet group High-B decreased significantly from both pre-drop and 5hr post-drop to 2.0±0.5 mM (Pre-drop: $p<0.0001$, 5hr: $p<0.0005$) at 2W post-drop, while the high salt diet group High-C decreased from pre-drop to 5hr post-drop ($p<0.0227$) and then further to 2W post-drop (Pre-drop: $p<0.0001$, 5hr: $p<0.0001$) (Appendix A, table A.5).

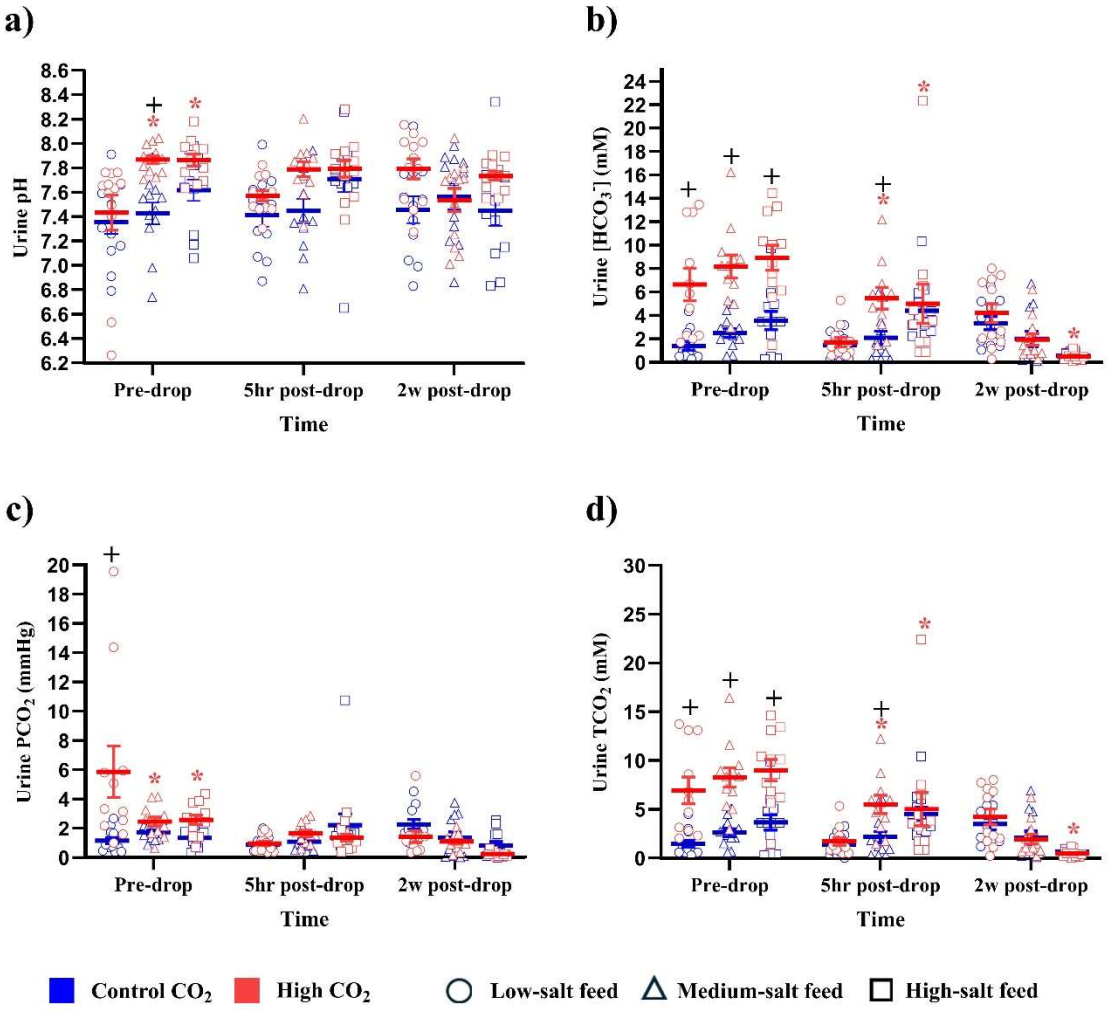


**Figure 7: Urine acid-base parameters of juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Within high $CO_2$ groups, red asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet. Pluss sign (+) indicates a significant difference between control and high $CO_2$ treatment within diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

## 3.1.3 Plasma ions

### *Sodium concentrations [$Na^+$](mM)*

Between experimental and control groups, the mean sodium concentrations were only significantly different low salt diet groups at 2W post-drop, where Control-A at 153.1±0.6 mM was slightly higher than High-A at 147.0±1.7 mM ($p<0.0486$, Fig. 8a). For experimental groups at 2W post-drop, sodium concentration was significantly lower for the low salt diet group High-A compared to both the medium salt diet group High-B at 155.4±1.4 mM ($p<0.0014$) and the high salt diet group High-C at 157.5±0.8 mM ($p<0.0001$) (Fig. 8a).

Sodium concentration changed significantly for all control groups, with the low salt diet group Control-A started at 147.5±1.2 mM pre-drop and increased to 153.1±0.6mM ($p<0.0096$) at 2W post-drop, the medium salt diet group Control-B was 144.8±1.6 mM at pre-drop and rose to 150.3±2.4 mM ($P<0.0134$) at 2W post-drop, while Control-C was significantly higher at 154.1±3.2 mM for 2W post-drop compared to 150.8±1.1 mM ($p<0.0225$) at pre-drop and 150.2±0.8 mM ($p<0.0267$) at 5hr post-drop (Appendix A, table A.6). Also, the experimental groups with medium and high salt diets changed significantly as well, with the medium salt diet group High-B being significantly higher at 155.4±1.4 mM for the 2W post-drop compared to pre-drop at 148.6±1.3 mM ($p<0.0316$) and 5hr post-drop at 147.8±0.8 mM ($p<0.0016$), while the high salt diet group High-C increasing to 157.5±0.8 mM for the 2W post-drop from 146.3±0.6 mM ($p<0.0001$) at pre-drop and 148,7±1.9 mM ($p<0.0042$) at 5hr post-drop (Appendix A, table A.6).

### *Chloride concentrations [$Cl^-$](mM)*

For mean chloride concentrations in experimental and control groups, the low salt diets at 5h post-drop, where control group Control-A at 114.4±2.3 mM was slightly higher than High-A at 106.6±2.0 ($p<0.0450$, Fig. 8b). Additionally for high salt diet groups at 2W post-drop, the experimental group High-C was 128.6±1.5 mM and significantly higher than the control group Control-C at 115.9±2.8 mM ($p<0.0001$, Fig. 8b).

For control groups, a significant difference in chloride concentration was found for the pre-drop sampling between the medium salt diet group Control-B at 110.2±2.4 mM and the high salt diet group Control-C at 119.3±1.4 ($p<0.0149$, Fig. 8b). For the 5hr post-drop, the experimental group with the high salt diet High-C had a mean chloride concentration of 118.1±2.5 mM and were significantly higher than both the low salt diet group High-A ($p<0.0003$) and the medium salt diet group High-B at 110.0±1.7 mM ($p<0.0277$) (Fig. 8b). At 2W post-drop, the experimental group with high salt diet High-C was 128.6±1.5 and had significantly higher chloride concentrations than both High-A at 115.1±1.6 ($P<0.0001$) and Control-C ($P<0.0001$) (Fig. 8b).

Only the control group with the low salt diet had a significant change to chloride concentration between sampling times, with Control-A increasing from 5hr post-drop to 121.2±0.9 mM ($p<0.0418$) at 2W post-drop (Appendix A, table A.6). The sodium concentration for the experimental groups changed significantly, with the low salt diet group High-A going from 5hr post-drop to 115.1±1.6 mM ($p<0.0030$) at 2W post-drop, the medium salt diet group High-B had increased to 121.7±1.2 mM at 2W post-drop from 112.8±1.9 mM ($p<0.0012$) pre-drop and

110.0±1.7 mM ($p<0.0001$) 5hr post-drop, while the high salt diet group High-C was significantly higher at 2W post-drop compared to 117.0±1.6 mM ($p<0.0004$) pre-drop and 118.1±2.5 mM ($p<0.0014$) 5hr post-drop (Appendix A, table A.6).

### *Potassium concentrations [$K^+$] (mM)*

Mean potassium concentration was only significantly different between experimental and control groups with the low salt diet at 5hr post-drop, where High-A was 2.3±0.2 mM while Control-A was 1.5±0.2 mM ($p<0.0112$, Fig.8c).

Between sampling times, the control group with low salt diet Control-A had significantly lower potassium concentration at 5hr post-drop compared to 2.0±0.2 mM ($p<0.0455$) pre-drop and 2.3±0.1 mM ($p<0.0014$) 2W post-drop (Appendix A, table A.6). For experimental groups, the low and medium salt diets changed significantly between sampling times, with High-A going from 1.8±0.1 at pre-drop and rising to 2.3±0.2 mM 5hr post-drop ($p<0.0235$) and 2.3±0.1 mM ($p<0.0235$) 2W post-drop, while High-B was 1.5±0.2 mM at pre-drop and increased to 2.1±0.2 ($p<0.0448$) at 2W post-drop (Appendix A, table A.6).

### *Magnesium concentrations [$Mg^{2+}$] (mM)*

Mean magnesium concentration was only significantly different between high salt diet groups at pre-drop, with the control group Control-C at 1.05±0.06 mM being significantly higher than the experimental group High-C at 0.76±0.04 mM ($p<0.0003$, Fig. 8d).

Additionally, only high salt diets had a significant change in plasma magnesium concentrations between sampling times, with Control-C being significantly lower pre-drop compared to 0.82±0.06 mM ($p<0.0401$) at 5hr post-drop and 0.73±0.07 mM ($p<0.0031$) for 2W post-drop, while High-C decreased significantly from 0.88±0.05 mM at 5hr post-drop to 0.74±0.04 mM ($p<0.0440$) at 2W post-drop (Appendix A, table A.6).

### *Calcium concentrations [$Ca^{2+}$] (mM)*

Experimental groups had significantly higher plasma calcium concentrations at pre-drop compared to control groups, with the experimental low salt diet group High-A being 3.06±0.03 mM compared to the control group Control-A at 2.73±0.05 mM ($p<0.0004$), the experimental medium salt diet group High-B was 3.05±0.04 mM compared to Control-B at 2.74±0.06 mM ($p<0.0009$), and the experimental high salt diet group High-C was 3.19±0.06 mM compared to Control-C at 2.70±0.05 mM ($p<0.0001$) (Fig. 8e). At 2W post-drop, the calcium concentration for the control group with medium salt diet was significantly lower than the low and high salt

diet groups, with Control-B being 2.63±0.09 mM while Control-A was 2.92±0.08 mM ($p<0.0028$) and Control-C was 2.90±0.09 mM ($p<0.0059$) (Fig. 8e).

Only the experimental groups had a significant change in calcium concentration over time, with the low salt diet group High-A decreasing from pre-drop and 2.91±0.05 mM at 5hr post-drop to 2.72±0.05 mM (Pre-drop: $p<0.0001$, 5hr: $p<0.0116$) at 2W post-drop, the medium salt diet group High-B decreased from pre-drop to 2.84±0.03 mM ($p<0.0004$) at 2W post-drop, while the high salt diet group High-C decreased from pre-drop to 2.96±0.05 mM ($p<0.0059$) at 5hr post-drop and then decreased further to 2.71±0.04 mM for 2W post-drop (Pre-drop: $p<0.0001$, 5hr: $p<0.0028$) (Appendix A, table A.6).

### *Phosphate concentrations [$PO_4^{3-}$] (mM)*

The only significant difference in mean plasma phosphate concentration was between experimental and control groups with low salt at 5h post-drop, with Control-A at 2.27±0.12 mM being higher than High-A at 1.76±0.05 mM ($p<0.0063$, Fig. 8f). For control groups at pre-drop, the high salt diet group Control-C at 2.94±0.011 mM was significantly higher than both the low and medium salt diet groups, with Control-A at 2.32±0.08 mM ($p<0.0005$) and Control-B at 2.40±0.09 mM ($p<0.0036$) (Fig. 8f). At pre-drop, the experimental group with the low salt diet High-A at 2.25±0.07 mM had significantly lower phosphate concentrations than the medium and high salt diet groups, with 2.71±0.09 mM ($p<0.0263$) for High-B and 2.84±0.12 mM ($p<0.0011$) for High-C (Fig. 8f). For the 5hr post-drop, the experimental low salt groups High-A was still significantly lower than the high salt group High-C at 2.57±0.18 mM ($p<0.0001$) (Fig. 8f). The phosphate concentrations for low salt groups was significantly lower than high salt groups at 2W post-drop, where the experimental group High-A was 2.26± 0.07 mM compared to High-C at 3.01±0.18 ($p<0.0001$), while the control group Control-A was 2.25± 0.06 mM compared to Control-C at 2.93±0.12 mM ($p<0.0001$) (Fig. 8f).

Phosphate concentrations only changed for experimental groups with low and medium salt diets, where High-A decreased from pre-drop to 5hr post-drop ($p<0.0001$) and then increased from 5hr post-drop to 2W post-drop ($p<0.0001$), while High-B decreased from pre-drop to 2.17±0.05 mM ($p<0.0001$) at 5hr post-drop and then increased from 5hr and 2W post-drop ($p<0.0003$) Appendix A, table A.6).

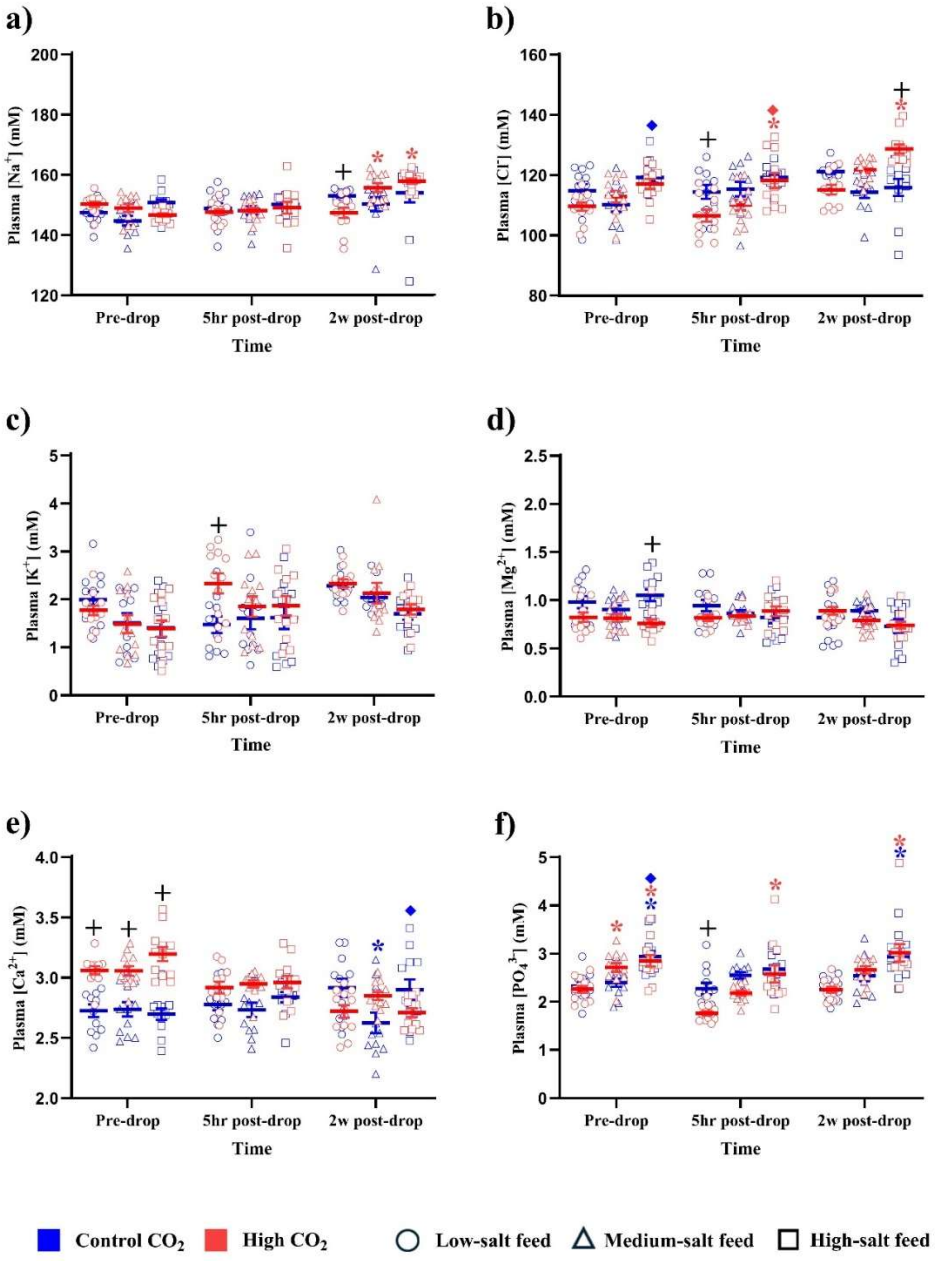


**Figure 8: Plasma ion concentrations of juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Within the control $CO_2$ groups, blue asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while blue diamond (◆) indicates a significant difference between medium and high salt diet. within the control $CO_2$ group. Within high $CO_2$ groups, red asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while red diamond shape (◆) indicates a significant difference between medium and high salt diet. Pluss sign (+) indicates a significant difference between control and high $CO_2$ treatment within diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

### 3.1.4 Urine ions

*Urine sodium concentration [$Na^+$] mM*

At pre-drop, the mean sodium concentration in urine was only significantly higher for the experimental group with low salt diet High-A at 33.6±2.7 mM, compared to the respective control group Control-A at 21.1±1.6 mM ($p<0.0388$, Fig. 9a).

Only the experimental groups with low and medium salt diets significantly changed between sampling times, with High-A decreasing from pre-drop to 24.6±1.1 mM ($p<0.0213$) at 5hr post-drop, while the medium salt diet group High-B decreased significantly to 19.6±0.9 at 2W post-drop from 29.7±2.9 mM ($p<0.0211$) at pre-drop and 27.8±5.3 mM ($p<0.0052$) at 5hr post-drop (Appendix A, table A.7).

*Urine chloride concentration [$Cl^-$] mM*

The mean urine chloride concentration was higher for the experimental groups compared to their respective control groups, but there was only a significant difference for low and medium salt groups at pre-drop. The experimental group with low salt diet High-A at 56.6±17.4 mM was significantly higher than the respective control group Control-A at 11.5±1.2 mM ($p<0.0001$), while the medium salt diet group High-B was 37.4±11.8 mM compared to Control-B at 13.2±0.9 mM ($p<0.0147$) (Fig. 9b).

Only group High-A had a significant change in chloride concentration, decreasing from pre-drop to 13.6±0.4 mM ($p<0.0021$) at 5hr post-drop, and then increasing to 21.1±4.1 mM ($p<0.0382$) at 2W post-drop (Appendix A, table A.7).

*Urine potassium concentration [$K^+$] mM*

The experimental groups with low and medium salt diets at pre-drop had significantly higher mean urine potassium concentrations than their respective control groups, with the low salt diet group High-A being 46.4±18.0 mM compared to Control-A at 1.5±0.2 mM ($p<0.0001$), and the medium salt diet group High-B was 24.2±12.1 mM compared to Control-B at 1.6±0.1 mM ($p<0.0161$) (Fig. 9c).

Only the experimental group with low salt diet had a significant change in potassium concentration between sampling times, with High-A being significantly higher at pre-drop compared to 2.4±0.2 mM ($p<0.0075$) at 5hr post-drop and 3.7±1.4 mM ($p<0.0256$) at 2W post-drop (Appendix A, table A.7).

### *Urine magnesium concentration [$Mg^{2+}$] mM*

The mean urine magnesium concentration was significantly different between experimental and control groups for medium and high salt diets at 2W post-drop, where the experimental group with medium salt diet High-B was 0.21±0.05 compared to the control group Control-B at 0.82±0.20 mM ($p<0.0093$), while the experimental group with high salt diet High-C was 1.23±0.22 mM compared to the control group Control-C 2.39±0.53 mM ($p<0.0043$) (Fig. 9d).

Additionally, at 2W post-drop the group High-B was significantly higher than the experimental group with low salt diet High-A at 0.68±0.21 mM ($p<0.0025$), while Control-C was higher than both the low salt diet group Control-A at 0.82±0.20 mM ($p<0.0046$) and the medium salt diet group Control-B at 2.39±0.53 mM ($p<0.0001$) (Fig. 9d).

All groups changed significantly between sampling times. For control groups, the low salt diet group Control-A increased from 0.77±0.31 mM at pre-drop to 1.22±0.24 mM ($p<0.0197$) 2W post-drop, the medium salt diet group Control-B was higher at 2W post-drop compared to 0.35±0.04 mM ($p<0.0029$) at pre-drop and 0.39±0.09 mM ($p<0.0481$) at 5hr post-drop, and the high salt diet group Control-C was also higher at 2W post-drop compared to 0.81±0.20 mM ($p<0.0074$) at pre-drop and 0.68±0.18 mM ($p<0.0038$) at 5hr post-drop (Appendix A, table A.7). For the experimental groups, the low salt diet group High-A decreased from 0.80±0.27 mM at pre-drop to 0.19±0.03 mM ($p<0.0018$) at 5hr post-drop and then increased to 0.68±0.21 mM ($p<0.0097$) at 2W post-drop, while the high salt diet group increased from 0.24±0.11 mM at 5hr post drop to the 2W post-drop ($p<0.0006$) sampling (Appendix A, table A.7).

### *Urine calcium concentration [$Ca^{2+}$] mM*

For groups with high salt diets at 2W post-drop, the mean urine calcium concentration was significantly higher for the experimental group High-C at 9.22±2.04 mM compared to the respective control group Control-C at 4.44±0.84 mM ($p<0.0001$, Fig. 9e).

Within control groups at 2W post-drop, the low salt diet group Control-A at 0.43±0.07 mM was significantly lower than both the medium salt diet group Control-B at 3.29±1.05 mM ($p<0.0041$) and Control-C ($p<0.0001$) (Fig. 9e). For experimental groups at 2W post-drop, the group High-C was significantly higher than both the low salt diet group High-A at 0.52±0.10 mM ($p<0.0001$) and the medium salt diet group High-B at 2.50±0.65 mM ($p<0.0001$) (Fig. 9e).

The mean urine calcium concentration changed significantly for all groups over time. For the control groups, Control-A had increased at the 2W post-drop from 0.23±0.09 mM ($p<0.0063$) at pre-drop, Control-B was higher at 2W post-drop compared to 0.23±0.03 mM ($p<0.0003$) at

pre-drop and 0.24±0.06 mM ($p<0.0003$) at 5hr post-drop, and Control-C was also higher at 2W post-drop compared to 0.27±0.06 mM ($p<0.0001$) at pre-drop and 0.31±0.08 mM ($p<0.0001$) at 5hr post-drop (Appendix A, table A.7). For experimental groups, High-A decreased from 0.27±0.04 mM at pre-drop to 0.06±0.01 mM ($p<0.0001$) at 5hr post-drop before rising to 0.52±0.10 mM (Pre-drop: $p<0.0290$, 5hr: $p<0.0001$) at 2W post-drop, High-B was higher at 2W post-drop compared to 0.27±0.06 mM ($p<0.0001$) at pre-drop and 0.21±0.01 mM ($p<0.0001$) at 5hr post-drop, while Control-C was also higher at 2W post-drop compared to 0.15±0.03 mM ($p<0.0001$) at pre-drop and 0.19±0.04 mM ($p<0.0001$) at 5hr post-drop (Appendix A, table A.7).

### *Urine phosphate concentration [$PO_4^{3-}$] mM*

The mean urine phosphate concentration was generally higher for control groups compared to experimental groups, except for low salt groups at pre-drop where the experimental group was higher than the control, albeit not significantly. The only significant difference was between the control and experimental groups was for medium salt diet groups at 2W post-drop, where Control-B at 5.5±0.9 mM was significantly higher than High-B at 2.1±0.4 mM ($p<0.0016$, Fig.9f).

Additionally, for the 2W post-drop sampling, the high salt groups were significantly higher than respective low salt groups, with Control-C at 6.6±0.9 mM being higher than Control-A at 3.3±1.0 mM ($p<0.0042$), while High-C at 4.5±0.5 mM was higher than High-A at 1.6±0.3 mM ($p<0.0098$) (Fig. 9f).

For control groups, only Control-C significantly changed between sampling times, having increased at 2W post-drop from 2.5±0.8 mM ($p<0.0007$) at pre-drop and 1.2±0.4 mM ($p<0.0001$) at 5hr post-drop (Appendix A, table A.7). For experimental groups, High-A was 0.2±0.1 at 5hr post-drop and significantly lower than 3.1±1.0 mM ($p<0.0001$) pre-drop and 2W post-drop ($p<0.0001$), High-B increased significantly to 2W post-drop from 0.9±0.3 mM ($p<0.0402$) at pre-drop and 0.5±0.1 mM ($p<0.0178$) at 5hr post-drop, while High-C also increased at 2W post-drop from 1.3±0.3 mM ($p<0.0004$) at pre-drop and 0.9±0.3 mM ($p<0.0001$) at 5hr post-drop (Appendix A, table A.7).

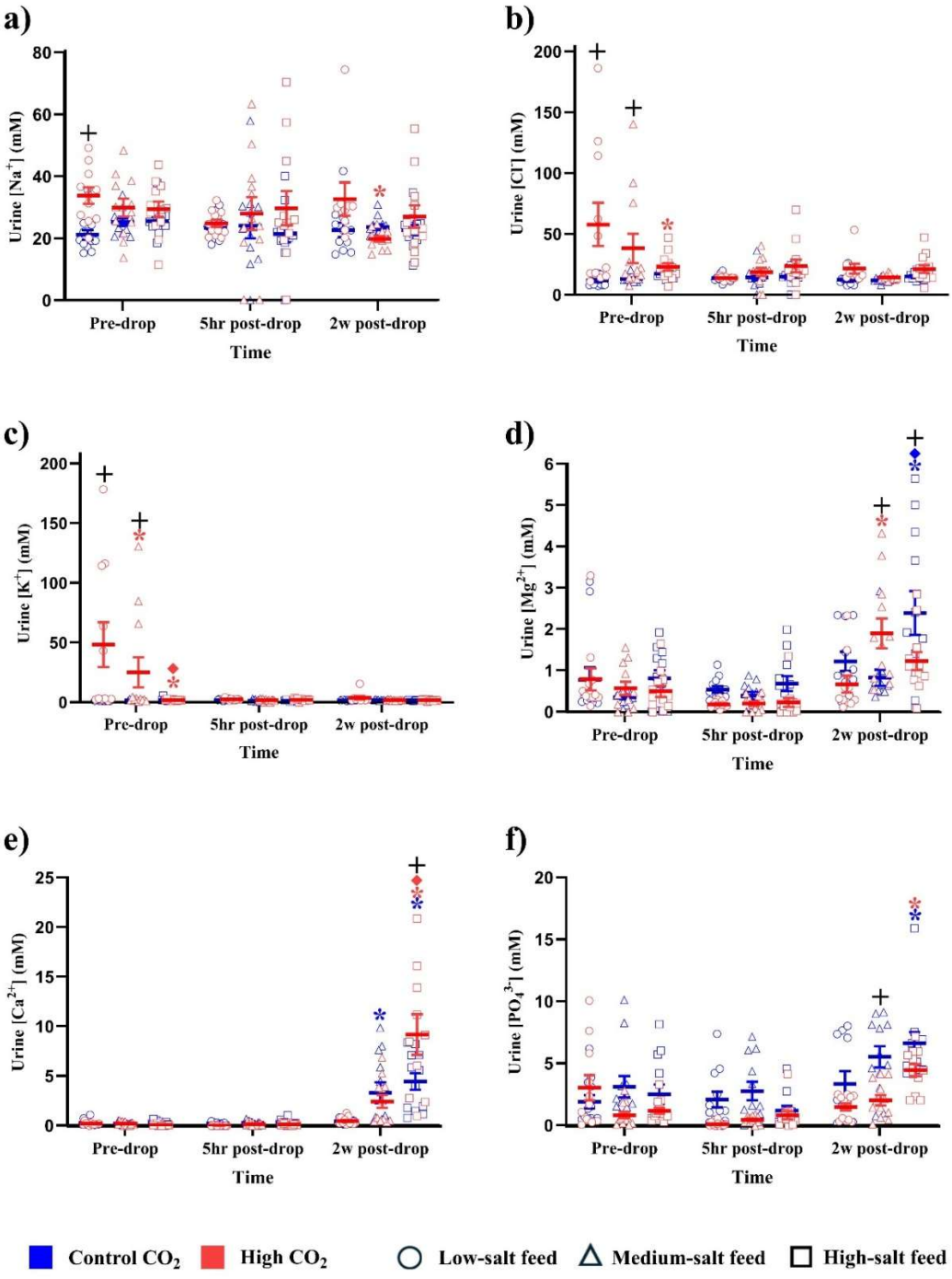


**Figure 9: Urine ion concentrations of juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Within the control $CO_2$ groups, blue asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while blue diamond (◆) indicates a significant difference between medium and high salt diet. within the control $CO_2$ group. Within high $CO_2$ groups, red asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while red diamond shape (◆) indicates a significant difference between medium and high salt diet. Pluss sign (+) indicates a significant difference between control and high $CO_2$ treatment within diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

### 3.1.5 Plasma metabolites

*Creatinine concentration (M)*

Creatinine concentration was only different between salt diet groups at 2W post-drop. For control groups, the high salt diet group Control-C at 26.2±1.3 M was significantly higher than both the low salt diet group Control-A at 10.9±2.6 M ($p<0.0001$) and the medium salt diet group Control-B at 16.17±2.8 M ($p<0.0259$) (Fig. 10a). For experimental groups, the high salt diet group High-C was 30.7±4.6 M and were significantly higher than the low salt diet group High-A at 13.8±3.9 M ($p<0.0001$) and the medium salt diet group High-B at 18.5±3.6 M ($p<0.0029$) (Fig. 10a).

The creatinine concentrations of the medium and high salt diet groups changed over time for both experimental and control groups. The control group with medium salt diet control-B decreased from 13.9±0.8 M pre-drop to 7.8±0.8 M ($p<0.0005$) 5hr post-drop and then increased from 5hr post-drop to 16.2±2.8 M ($p<0.0001$) at 2W post-drop, while the high salt diet group Control-C was higher at 2W post-drop than pre-drop at 14.6±0.6 M ($p<0.0001$) and 5hr post-drop at 16.9±0.5 M ($p<0.0001$) (Appendix A, table A.6). For the experimental groups, the medium salt diet group High-B was higher at 2W post-drop than pre-drop at 9.4±1.3 M ($p<0.0180$) and 5hr post-drop at 8.7±0.7 M ($p<0.0098$), and the high salt diet group High-C was higher at 2W post-drop compared to pre-drop at 10.4±0.6 M ($p<0.0029$) and 5hr post-drop at 12.8±4.4 M ($p<0.0005$) (Appendix A, table A.6).

*Plasma albumin (M)*

The mean plasma albumin concentrations was higher for experimental groups with low and medium salt diets compared to respective control groups at 5hr post-drop, with the experimental group High-A at 271.3±5.8 M being significantly higher than the control group Control-A at 236.2±8.0 M ($p<0.0002$), and the experimental group High-B was 257.6±4.3 M and significantly higher than the control group Control-B at 228.6±4.8 M ($p<0.0038$) (Fig. 10b).

Albumin concentrations only significantly changed between sampling times for the experimental groups, with the low salt diet group High-A starting at 251.4±4.2 M for pre-drop increasing to 271.3±5.9 M ($p<0.00246$) for 5hr post-drop and then decreasing to 225.3±5.1 M (Pre-drop: $p<0.0027$, 5hr: $p<0.0001$) for 2W post-drop, the medium salt diet group High-B was 217.1±4.7 M at 2W post-drop and were significantly lower than 245.4±6.8 M ($p<0.0021$) at pre-drop and 257.6±4.3 M ($p<0.0001$) at 5hr post-drop, while the high salt diet group High-C was 222.3±4.6 M at 2W post-drop which was significantly lower than 246.1±4.8 M ($p<0.0036$) at pre-drop and 256.4±4.9 M ($p<0.0001$) at 5hr post-drop (Appendix A, table A.6).

*Plasma total protein (g/L)*

The mean plasma total protein concentration at pre-drop was higher for the experimental groups compared to control groups, but only significant for the low salt diet group High-A at 30.1±1.6 g/L compared to the respective control group Control-A at 22.1±0.6 g/L ($p<0.0002$, Fig. 10c). At 5hr post-drop, the control group with low salt diet Control-A at 34.6±1.2 g/L had significantly higher protein concentration than High-A at 25.5±0.6 g/L ($p<0.0001$), while the experimental group with high salt diet High-C at 39.0±0.8 g/L were significantly higher than Control-C at 33.0±0.7 g/L ($p<0.0151$) (Fig. 10c).

Within the control groups at pre-drop, the low salt diet group Control-A was significantly lower than the medium salt diet group Control-B at 30.2±2.0 g/L ($p<0.0001$), while the high salt diet group Control-C at 37.9±0.8 g/L was higher than both Control-A ($p<0.0001$) and Control-B ($p<0.0003$) (Fig. 10c). Additionally, for 2W post-drop Control-C at 37.1±1.1 g/L was significantly higher than Control-A at 30.9±0.7 g/L ($p<0.0074$, Fig. 10c). For the experimental groups at pre-drop, the high salt diet group High-C at 39.6±0.8 g/L had significantly higher protein concentrations compared to the low salt diet group High-A at ($p<0.0001$) and the medium salt diet group High-B at 33.6±2.4 g/L ($p<0.0111$) (Fig.10c). At the 5hr post-drop, High-A was significantly lower than High-B at 31.4±2.2 g/L ($p<0.0115$), while High-C was significantly higher than both High-A ($p<0.0001$) and High-B ($p<0.0007$) (Fig .10c).

The mean plasma total protein concentration significantly changed between sampling times for low and high salt diet groups. For control groups, Control-A increased from pre-drop to 5hr post-drop ($p<0.0001$) and then decreased for 2W post-drop (Pre-drop: $p<0.0001$, 5hr: $p<0.0138$), while Control-C decreased from pre-drop to 5hr post-drop ($p<0.0010$) before increasing to 2W post-drop ($p<0.0059$) (Appendix A, table A.6). For experimental groups, High-A decreased from pre-drop to 5hr post-drop ($p<0.0137$) and increased from 5hr post-drop to 31.9±0.8 g/L for 2W post-drop ($p<0.0006$), while High-C had decreased significantly 2W post-drop at 32.8±0.8 g/L compared to pre-drop ($p<0.0014$) and 5hr post-drop ($p<0.0056$) (Appendix A, table A.6).

*Plasma glucose concentration (mM)*

Between groups, the mean plasma glucose concentration was only significantly different between low and high salt diet control groups at 2W post-drop, with the low salt diet group Control-A at 5.5±0.3 mM being slightly lower than the high salt diet group Control-C at 8.2±1.5 mM ($p<0.0437$, Fig. 10d).

For control groups, the glucose concentration significantly changed for the low and medium salt diet groups, with Control-A being 7.9±0.8 mM at pre-drop and having decreased at 2W post-drop ($p<0.0373$), while Control-B started at 9.4±1.0 mM and decreased to 6.3±0.5 mM ($p<0.0188$) at 2W post-drop (Appendix A, table A.6). For experimental groups, glucose concentration changed for all salt diet groups, with the low salt diet High-A changing from 8.6±0.6 mM at 5hr post-drop to 6.7±0.7 mM ($p<0.0282$) at 2W post-drop, the medium salt diet group High-B decreasing significantly from 7.8±0.4 mM at 5hr post-drop to 6.1±0.3 mM ($p<0.0341$) 2W post-drop, while the high salt diet High-C was 5.5±0.3 mM at 2W post-drop and were lower than both 6.7±0.4 mM at pre-drop ($p<0.0416$) and 8.3±1.0 mM at 5hr post-drop ($p<0.0049$) (Appendix A, table A.6).

*Plasma lactic acid (mM)*

There were no significant differences in mean plasma lactic acid concentrations between groups (Fig. 10e). Between sampling times, only the experimental low salt diet group changed significantly, decreasing from 2.06±0.35 mM at 5hr post-drop to 0.95±0.25 mM (<0.0358) at 2W post-drop (Appendix A, table A.6).

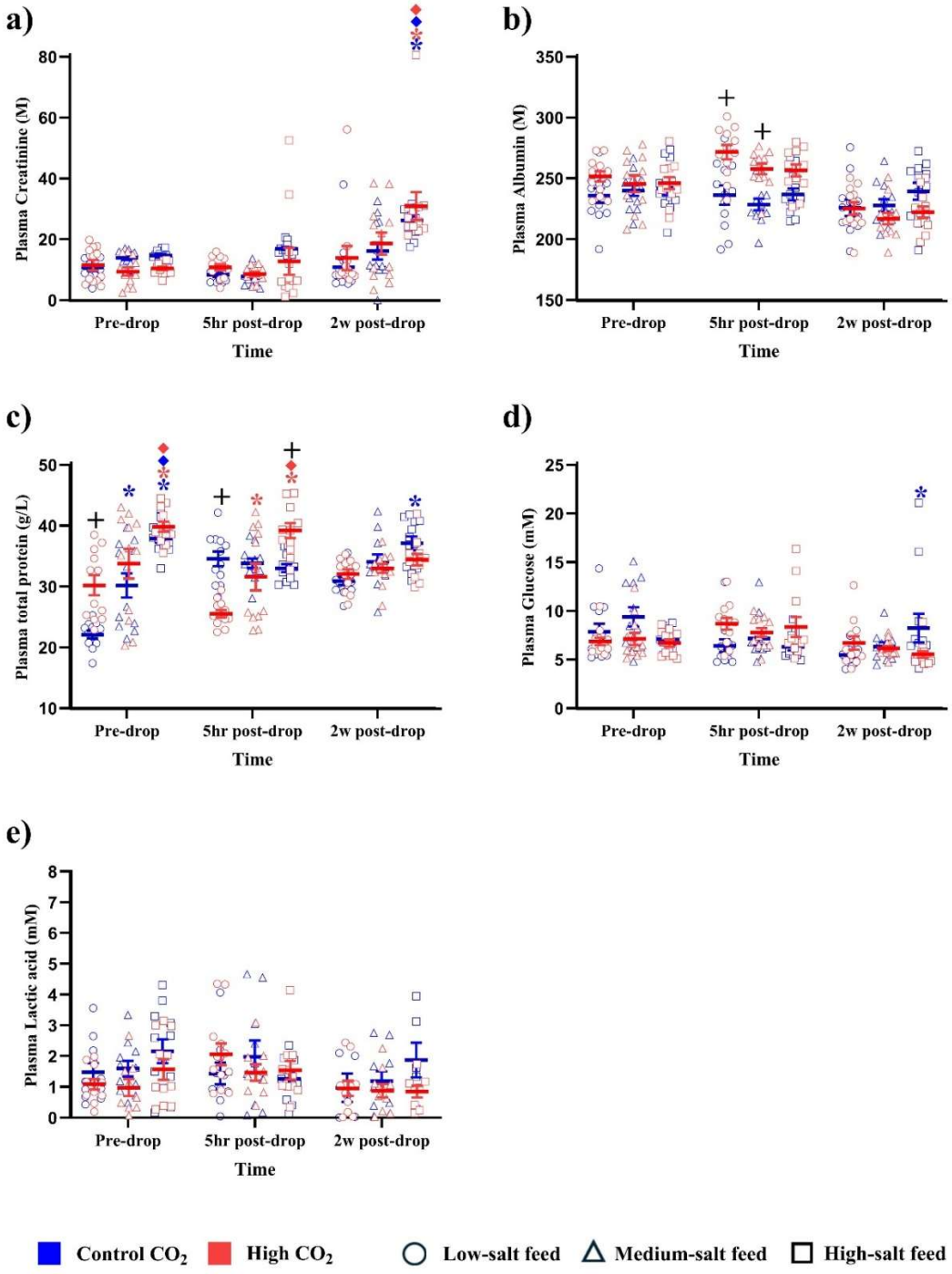


**Figure 10: Plasma metabolite concentrations of juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Within the control $CO_2$ groups, blue asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while blue diamond (◆) indicates a significant difference between medium and high salt diet. within the control $CO_2$ group. Within high $CO_2$ groups, red asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while red diamond shape (◆) indicates a significant difference between medium and high salt diet. Pluss sign (+) indicates a significant difference between control and high $CO_2$ treatment within diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

### 3.1.6 Urine metabolites

*Urine creatinine concentration (M)*

There were no significant differences in mean urine creatinine concentrations between experimental and control groups or between salt diet groups within sampling times (Fig. 11a).

The creatinine concentration significantly changed between sampling times for control groups with low and high salt diets, with Control-A being significantly lower at 27.6±2.4 M for the 5hr post-drop compared to 57.9±6.7 M ($p<0.0005$) at 2W post-drop ($p<0.0036$), while Control-C was significantly lower at 38.2±14.5 M for the 5hr post-drop compared to 62.4±3.8 M ($p<0.0013$) at pre-drop and to 56.4±5.5 M ($p<0.085$) at the 2W post-drop (Appendix A, table A.7). For experimental groups, the mean creatinine concentration significantly changed for the low salt diet group High-A being significantly lower at 24.6±3.7 M for the 5hr post-drop compared to 76.8±30.6 M ($p<0.028$) at pre-drop and to 200.2±116.6 M ($p<0.0023$) at the 2W post-drop (Appendix A, table A.7).

*Urine Albumin (M)*

There were no significant differences in mean urine albumin concentration between experimental and control groups within sampling times. At 5hr post-drop, the experimental group with medium salt diet High-B at 17.5±6.2 M was significantly higher than both the low salt diet group High-A at 5.4±1.5 M ($p<0.0353$) and the high salt diet group High-C at 2.9±10 M ($p<0.0050$) (Fig. 11b). Additionally, for the 2W post drop, High-A was 17.0±8.7 M and were significantly higher than High-B at 4.4±0.8 M ($p<0.0293$) and High-C at 0.9±0.2 M ($p<0.0017$) (Fig. 11b).

Only the high salt diet groups had a significant change in albumin concentration between sampling times, with control group Control-C having increased to 4.8±1.4 M at 2W post-drop from 1.2±0.5 M ($p<0.0123$) at pre-drop and 2.0±1.3 M ($p<0.0054$) at 5hr post-drop, while the experimental group High-C had decreased at 2W post-drop from 1.5±0.7 M ($p<0.0043$) pre-drop and 5hr post-drop ($p<0.0010$) (Appendix A, table A.7).

*Urine total protein (g/L)*

There were no significant differences in mean urine total protein between experimental and control groups or between salt diet groups within sampling times (Fig. 11c).

Between sampling times, only the high salt diet groups had a significant change in total protein concentration, with the control group Control-C having decreased from 4.0±0.6 g/L at pre-drop to 1.9±0.3 g/L ($p<0.0260$) at 5hr post-drop and 0.5±0.2 g/L ($p<0.0287$) at 2W post-drop, while

the experimental group High-C was 0.9±0.3 g/L at 2W post-drop and had decreased from 2.7±0.4 g/L ($p<0.0089$) at pre-drop and 2.6±0.6 g/L ($p<0.0340$) at 5hr post-drop (Appendix A, table A.7).

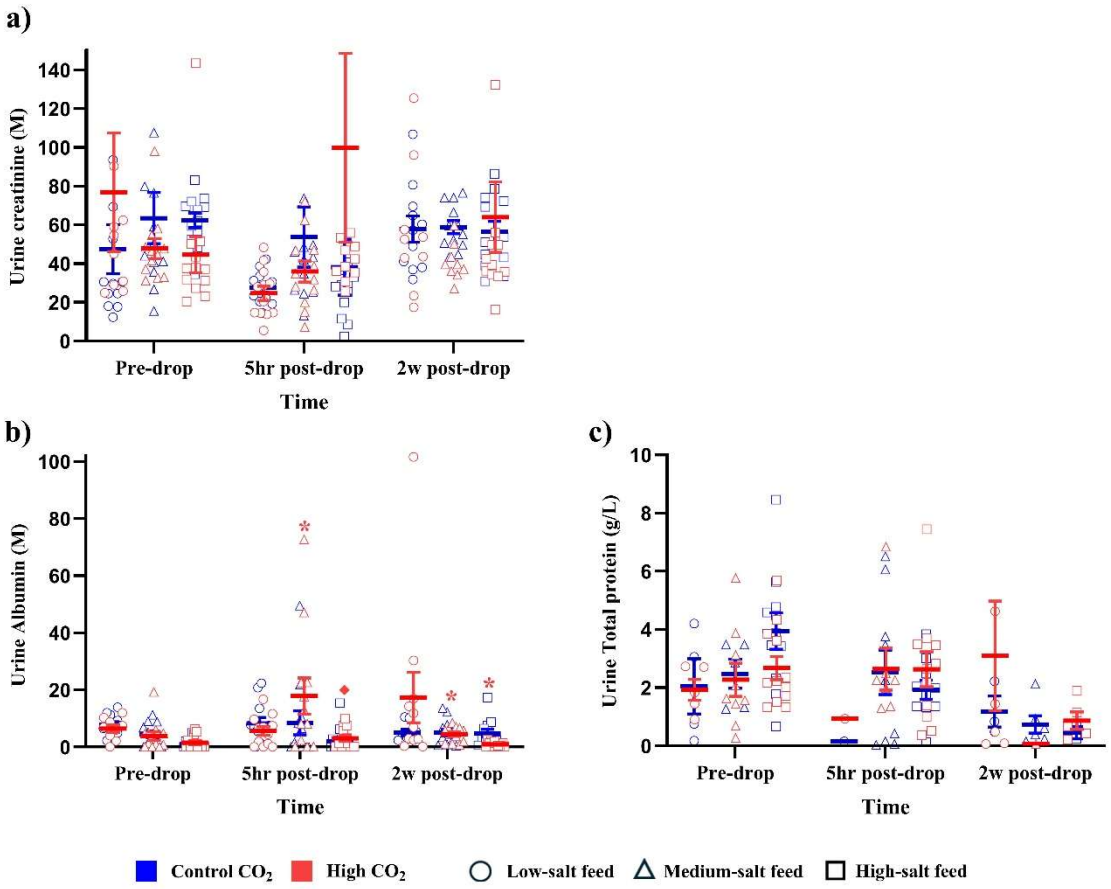


**Figure 11: Urine metabolite concentrations of juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Within high $CO_2$ groups, red asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while red diamond shape (◆) indicates a significant difference between medium and high salt diet. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

## 3.1.7 Urine plasma ratios

### *Sodium [$Na^+$] urine-plasma (UP) ratio*

Mean sodium UP ratio was only significantly different between low salt diets at pre-drop, with the experimental group High-A being 0.23±0.02 compared to the control group Control-A at 0.14±0.01 ($p<0.0271$, Fig. 12a). Only experimental groups with low and medium salt diets changed significantly between sampling times, where the low salt diet group High-A decreased from pre-drop to 0.17±0.01 ($p<0.0369$) at 5hr post-drop, while the medium salt diet group High-

B decreased to 0.13±0.01 at 2W post-drop compared to 0.20±0.02 ($p<0.0317$) at pre-drop and 0.22±0.03 ($p<0.0046$) at 5hr post-drop (Appendix A, table A.8).

### *Chloride [$Cl^-$] urine-plasma (UP) ratio*

Mean chloride UP ratio generally higher for experimental groups compared to control groups at pre-drop, with the experimental low salt diet group High-A at 0.52±0.17 being significantly higher than the respective control group Control-A at 0.10±0.01 ($p<0.0001$), and the experimental medium salt diet group High-B at 0.34±0.11 being significantly higher than the respective control group Control-B at 0.12±0.01 ($p<0.0322$) (Fig. 12b). Additionally, for experimental groups at pre-drop, the high salt diet group High-C at 0.19±0.03 was significantly lower than High-A ($p<0.0001$) (Fig. 12b).

Only experimental groups with low and medium salt diets changed significantly between sampling times, where High-A decreased from pre-drop to 0.13±0.01 ($p<0.0095$) at 5hr post-drop, while the medium salt diet group High-B decreased to 0.12±0.01 at 2W post-drop compared to pre-drop ($p<0.0331$) and 0.20±0.02 ($p<0.0122$) at 5hr post-drop (Appendix A, table A.8).

### *Potassium [$K^+$] urine-plasma (UP) ratio*

The mean potassium UP ratio was significantly higher from experimental groups with low and medium salt diets compared to their respective control groups, with the experimental low salt diet group High-A at 24.5±9.9 was higher than the control group Control-A at 0.8±0.1 ($p<0.0029$), while the experimental medium salt group High-B was 27.7±15.2 compared to the control group Control-B at 1.2±0.2 ($p<0.0008$) (Fig. 12c). Additionally, for experimental groups at pre-drop, the high salt diet group High-C at 1.7±0.4 was significantly lower than both High-A ($p<0.0048$) and High-B ($p<0.0005$) (Fig. 12c).

Between sampling times, Control-A had a significant change in mean UP ratio, increasing from pre-drop to 1.3±0.2 ($p<0.0306$) at 5hr post-drop and then decreased to 0.7±0.1 ($p<0.0169$) at 2W post-drop. For experimental groups with low and medium salt diets, UP ratio changed significantly between sampling times, whit High-A being significantly higher at pre-drop compared to 1.1±0.1 ($p<0.0015$) at 5hr post-drop and 1.6±0.7 ($p<0.0042$) at 2W post-drop, while High-B significantly decreased from pre-drop to 0.9±0.1 ($p<0.0063$) at 2W post-drop (Appendix A, table A.8).

### *Magnesium [$Mg^{2+}$] urine-plasma (UP) ratio*

The mean magnesium UP ratio was significantly different between experimental and control groups with medium and high salt diets at 2W post-drop, with the experimental medium salt diet group High-B at 2.53±0.50 and higher than the control group Control-B at 0.95±0.25 ($p<0.0402$), while the experimental high salt diet group High-C at 1.70±0.31 was lower than the control group Control-C at 4.20±1.24 ($p<0.0001$) (Fig. 12d). Additionally, for the 2W post-drop, Control-C was significantly higher than both the low salt diet group Control-A at 1.51±0.27 ($p<0.0001$) and Control-B ($p<0.0001$), while High-B was significantly higher than the experimental low salt diet group High-A at 0.76±0.24 ($p<0.0178$) (Fig. 12d).

For control groups, magnesium UP ratios changed significantly for Control-A increasing from 0.84±0.35 at pre-drop to 2W post-drop ($p<0.0093$), while Control-B increased from 0.39±0.05 at pre-drop to 0.64±0.05 ($p<0.0287$) at 5hr post-drop and 2W post-drop ($p<0.0015$) (Appendix A, table A.8). For experimental groups, High-A was 1.04±0.38 at pre-drop and decreased to 0.24±0.03 ($p<0.0019$) at 5hr post-drop then rising to 2W post-drop ($p<0.0215$), High-B increased at 2W post-drop from 0.86±0.19 ($p<0.0037$) at pre-drop and from 0.35 ($p<0.0001$) 5hr post-drop, while High-C increased at 2W post-drop from 0.78±0.23 ($p<0.0404$) at pre-drop and from 0.35±0.13 ($p<0.0001$) 5hr post-drop (Appendix A, table A.8).

### *Calcium [$Ca^{2+}$] urine-plasma (UP) ratio*

The mean calcium UP ratio was significantly different between experimental and control groups at 2W post-drop for high salt diet groups, with the experimental group High-C at 3.17±0.60 being significantly higher than the control group Control-C at 1.53±0.29 ($p<0.0001$, Fig 12e). At 2W post drop for control groups, the low salt diet group Control-A was 0.15±0.02 and were significantly lower than both the medium salt diet group Control-B at 1.34±0.44 ($p<0.0007$) and Control-C ($p<0.0001$) (Fig. 12e). For experimental groups at 2W post-drop, High-C was significantly higher than both the low salt diet group High-A at 0.19±0.04 ($p<0.0001$) and the medium salt diet group High-B at 0.90±0.24 ($p<0.0001$) (Fig. 12e).

For control groups, the calcium UP ratio was significantly different between sampling times, with Control-A increasing from 0.08±0.03 at pre-drop to 2W post-drop ($p<0.0079$), Control-B increased at 2W post-drop from 0.09±0.01 ($p<0.0004$) at pre-drop and 0.10±0.02 ($p<0.0003$) at 5hr post-drop, while Control-C increased at 2W post-drop from 0.10±0.02 ($p<0.0001$) at pre-drop and 0.11±0.03 ($p<0.0001$) at 5hr post-drop (Appendix A, table A.8). For experimental groups, High-A was 0.08±0.01 at pre-drop and decreased to 0.02±0.004 ($p<0.0001$) at 5hr post-drop then rising to 2W post-drop (Pre-drop: $p<0.0079$, 5hr: $p<0.0001$), High-B increased at 2W

post-drop from 0.09±0.01 ($p<0.0001$) at pre-drop and from 0.07±0.02 ($p<0.0001$) 5hr post-drop, while High-C increased at 2W post-drop from 0.05±0.01 ($p<0.0001$) at pre-drop and from 0.06± 0.01 ($p<0.0001$) 5hr post-drop (Appendix A, table A.8).

### *Phosphate [$PO_4^{3-}$] urine-plasma (UP) ratio*

The mean phosphate UP ratio was significantly higher for the control group with medium salt diet Control-B at 2.27±0.38 compared to the respective experimental group High-B at 0.81±0.17 ($p<0.0016$, Fig. 12f) for the 2W post-drop. Additionally, at pre-drop, High-B at 0.34±0.10 was significantly lower than the experimental low salt diet group High-A at 1.44±0.48 ($p<0.0466$, Fig. 12f)

Between sampling times, the control group with high salt diet Control-C significantly increased to 2.32±0.36 at 2W post-drop from 0.80±0.24 ($p<0.0004$) at pre-drop and from 0.42±0.12 ($p<0.0001$) at 5hr post-drop (Appendix A, table A.8). For experimental groups, High-A decreased from pre-drop to 0.11±0.03 ($p<0.0001$) at 5hr post-drop then rising to 0.70±0.13 ($p<0.0004$) at 2W post-drop, High-B increased at 2W post-drop from 0.34±0.10 ($p<0.0148$) at pre-drop and from 0.26±0.04 ($p<0.0044$) 5hr post-drop, while High-C increased to 1.54±0.17 at 2W post-drop from 0.43±0.08 ($p<0.0001$) at pre-drop and from 0.31±0.08 ($p<0.0001$) 5hr post-drop (Appendix A, table A.8).

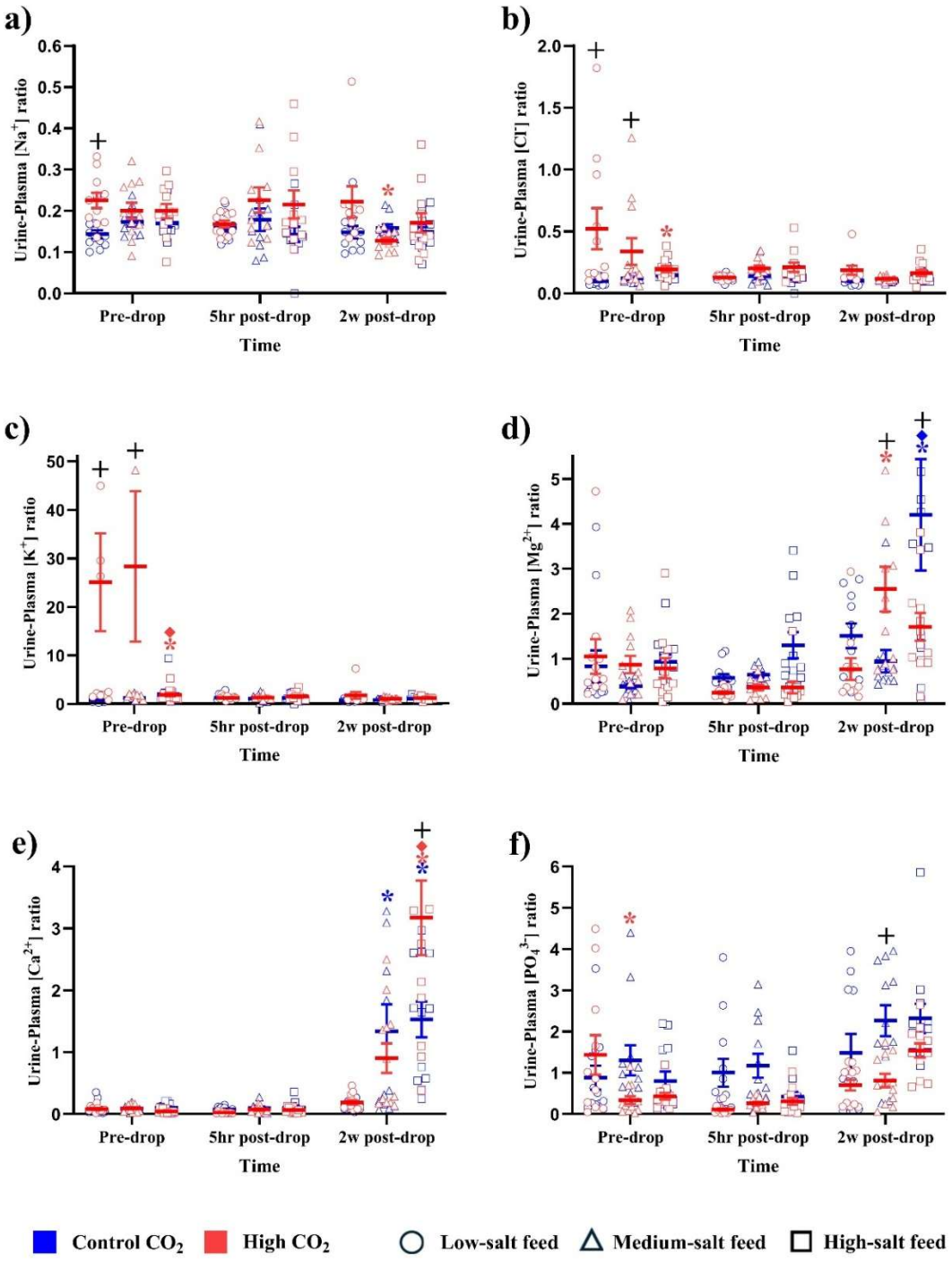


**Figure 12: Ion urine-plasma (UP) ratios for juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Within the control $CO_2$ groups, blue asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while blue diamond (◆) indicates a significant difference between medium and high salt diet. within the control $CO_2$ group. Within high $CO_2$ groups, red asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while red diamond shape (◆) indicates a significant difference between medium and high salt diet. Pluss sign (+) indicates a significant difference between control and high $CO_2$ treatment within diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

*Bicarbonate [$HCO_3^-$] urine-plasma (UP) ratio*

The mean bicarbonate UP ratio was only significantly different between experimental and control groups with medium salt diet at pre-drop, with the experimental group High-B being 0.37±0.04 compared to the control group Control-B at 0.15±0.02 ($p<0.0301$, Fig. 13a). At 5hr post-drop, the control group with high salt diet Control-C was 0.36±0.06 and was significantly higher than both the low salt diet group Control-A at 0.10±0.02 ($p<0.0028$) and Control-B at 0.16±0.04 ($p<0.0466$), while the experimental group with high salt diet High-C was 0.31±0.1 and were significantly higher than the low salt diet group High-A at 0.09±0.02 ($p<0.0282$) (Fig. 13a). At 2W post-drop, Control-C was 0.04±0.01 and were significantly lower than Control-A at 0.31±0.05 ($p<0.0038$, Fig. 13a), while High-C was 0.05±0.01 and had significantly lower bicarbonate UP ratio compared to High-A at 0.35±0.07 ($p<0.0003$, Fig. 13a).

Between sampling times, the bicarbonate UP ratio for Control-A was significantly lower for the 5hr post-drop compared to both pre-drop at 0.27±0.06 ($p<0.0395$) and the 2W post-drop ($p<0.0112$), while Control-C had decreased significantly at 2W post-drop from both 0.24± 0.05 ($p<0.0178$) at pre-drop and from 5hr post-drop ($p<0.0001$) (Appendix A, table A.8). For experimental groups, High-A was significantly lower at 5hr post-drop compared to 0.28±0.05 ($p<0.0334$) for pre-drop and the 2W post-drop ($p<0.0021$), High-B decreased significantly to 0.18±0.05 at 2W post-drop from pre-drop ($p<0.0024$) and 0.28±0.06 ($p<0.0282$) at 5hr post-drop, while High-C decreased significantly at 2W post-drop from 0.36±0.04 ($p<0.0001$) at pre-drop and 5hr post-drop ($p<0.0001$) (Appendix A, table A.8).

*Partial preassure $CO_2$ urine-plasma (UP) ratio*

The mean partial pressure $CO_2$ was only significantly different between the experimental and control groups for low salt diets at pre-drop, where the experimental group High-A was 0.39±0.137 and higher than the control group Control-A at 0.11±0.025 ($p<0.0005$, Fig. 13b). For experimental groups at pre-drop, High-A was significantly higher than both the medium salt diet group High-B at 0.14±0.022 ($p<0.0035$) and the high salt diet group High-C at 0.14±0.024 ($p<0.0030$) (Fig. 13b). Additionally, for control groups at 2W post-drop, Control-A was 0.33±0.065 and were significantly higher than the high salt diet group Control-C at 0.08±0.026 ($p<0.0030$, Fig. 13b).

Control-A was the only control group that had a significant change in partial preassure $CO_2$ between sampling times, with an increase at 2W post-drop from both pre-drop ($p<0.0006$) and 5hr post-drop at 0.06±0.014 ($p<0.0001$) (Appendix A, table A.8). For experimental groups, High-A was significantly lower at 0.06±0.010 for the 5hr post-drop compared to pre-drop

($p<0.0003$) and 0.19±0.070 at 2W post-drop ($p<0.0292$), while High-C decresed signicantly to 0.02±0.004 at 2W post-drop from pre-drop ($p<0.0001$) and 0.12±0.019 ($p<0.0001$) at 5hr post-drop (Appendix A, table A.8).

### *Total $CO_2$ urine-plasma (UP) ratio*

The experimental and control groups with medium salt diets at pre-drop was the only groups with a significant difference in mean total $CO_2$ UP ratio, with the experimental group High-B being 0.35±0.04 and significantly higher than the control group Control-B at 0.15±0.02 ($p<0.0205$, Fig. 13c). The control group with high salt diet Control-C was significantly higher than both the low salt diet group Control-A at 0.09±0.02 ($p<0.0011$) and the medium salt diet group Control-B at 0.16±0.04 ($p<0.0283$) for the 5hr post-drop, while Control-C at 0.04±0.01 was significantly lower than Control-A at 0.31±0.05 ($p<0.0013$) for the 2W post-drop (Fig. 13c). For experimental groups, at 5hr post-drop the low salt diet group High-A at 0.09±0.02 was significantly lower than both High-B at 0.30±0.06 ($p<0.0194$) and the high salt diet group High-C at 0.30±0.10 ($p<0.0184$), while at 2W post-drop High-A was 0.35±0.07 and were significantly higher than High-C at 0.04±0.01 ($p<0.0001$) (Fig. 13c).

The mean total $CO_2$ UP ratio changed between sampling times for the control groups with low and high salt diets, where Control-A significantly increased at 2W post-drop from 0.10±0.03 ($p<0.0005$) at pre-drop and 5hr post-drop ($p<0.0003$), while Control-C significantly decreased at 2W post-drop from 0.23±0.05 ($p<0.0189$) at pre-drop and 5hr post-drop ($p<0.0001$) (Appendix A, table A.8). For experimental groups, High-A was significantly lower at 5hr post-drop compared to 0.28±0.05 ($p<0.0215$) at pre-drop and 2W post-drop ($p<0.0017$), High-B decreased significantly to 0.17±0.05 at 2W post-drop from 0.35±0.04 ($p<0.0030$) at pre-drop and 0.30±0.01 ($p<0.0337$) at 5hr post-drop, while High-C significantly decreased at 2W post-drop from 0.35±0.04 ($p<0.0001$) at pre-drop and 5hr post-drop ($p<0.0001$) (Appendix A, table A.8).

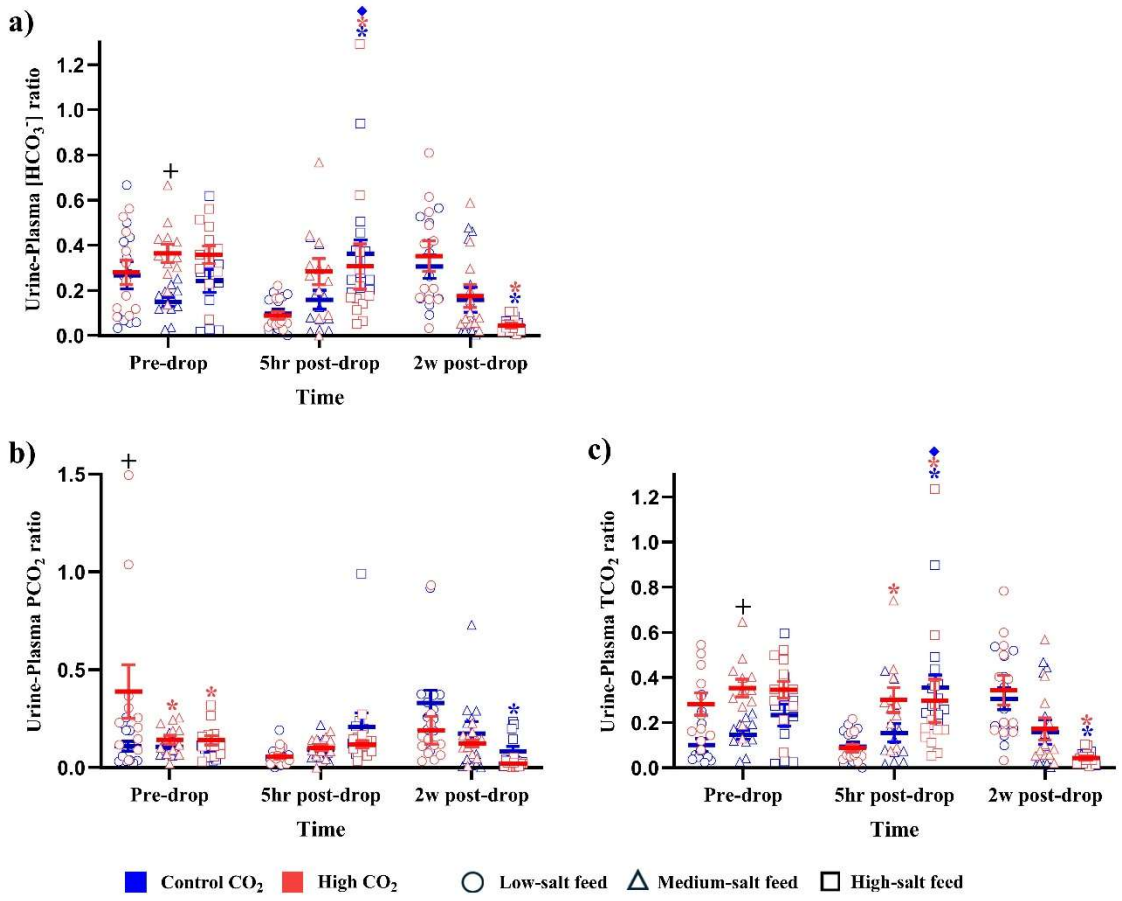


**Figure 13: Acid-base urine-plasma (UP) concentration ratios for juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Within the control $CO_2$ groups, blue asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while blue diamond (◆) indicates a significant difference between medium and high salt diet. within the control $CO_2$ group. Within high $CO_2$ groups, red asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet. Pluss sign (+) indicates a significant difference between control and high $CO_2$ treatment within diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

### *Albumin urine-plasma (UP) ratio*

The mean albumin UP ratio was only significantly different between experimental groups, with the high salt diet group High-C being 0.011±0.004 at 5hr post-drop and significantly lower than the medium salt diet group High-B at 0.069±0.025 ($p<0.0105$), while at the 2W post-drop High-C was significantly lower than the low salt diet group High-A at 0.024±0.004 ($p<0.0051$) (Fig. 14a).

Only High-C had a significant change in albumin UP ratio between sampling times, going from 0.017±0.003 at pre-drop to 0.004±0.001 at 2W post-drop ($p<0.0080$, Appendix A, table A.8).

*Creatinine urine-plasma (UP) ratio*

For the mean creatinine UP ratio at 5hr post-drop, the experimental group with high salt diet High-C was 10.9±2.7 and were significantly higher than both the control group with high salt diet Control-C at 2.1±0.7 ($p<0.0093$) and the experimental group with low salt diet High-A at 2.3±0.3 ($p<0.0111$) (Fig. 14b).

For control groups, only Control-C changed between sampling times with pre-drop at 4.3±0.3 being significantly higher than both 5hr post-drop ($p<0.0003$) and the 2W post-drop at 2.3±0.3 ($p<0.0058$) (Appendix A, table A.8). For experimental groups, High-A significantly increased from 5hr post-drop to 10.4±2.7 ($p<0.0009$) at 2W post-drop, while High-C decreased significantly to 2.0±0.3 at 2W post-drop from 4.4±1.1 ($p<0.0252$) at pre-drop and from 5hr post-drop ($p<0.0005$) (Appendix A, table A.8).

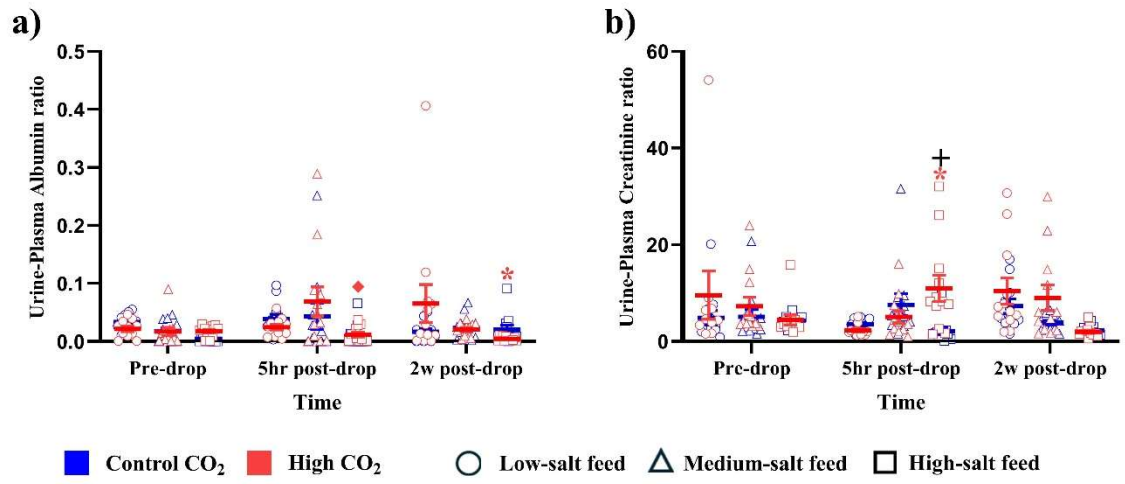


**Figure 14: Albumin and creatinine urine-plasma (UP)concentration ratios for juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Within high $CO_2$ groups, red asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while red diamond shape (◆) indicates a significant difference between medium and high salt diet. Pluss sign (+) indicates a significant difference between control and high $CO_2$ treatment within diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

### 3.1.8 Urine Plasma fractional excretion rate (%)

*Sodium [$Na^+$] urine-plasma fractional excretion (FE) rate (%)*

At 5hr post-drop, the control group with high salt diet Control-C was 18.0±8.2 and were significantly higher than the respective experimental group High-C at 3.8±1.3 ($p<0.0001$), as well as the control group with low salt diet Control-A at 5.2±0.7 ($p<0.0006$) and the control group with medium salt diet Control-B at 3.8±0.9 ($p<0.0002$) (Table 3).

Between sampling times for control groups, the sodium FE rate for Control-A significantly decreased from 5hr post-drop to 3.6±1.5 ($p<0.0385$) at 2W post-drop, while Control-C increased significantly from pre-drop at 4.1±0.3 to 5hr post-drop ($p<0.0009$) and 7.5±0.9 ($p<0.0288$) at 2W post-drop (Appendix A, table A.9). For experimental groups, the sodium FE rate for the low salt diet group High-A significantly decreased from 8.4±0.7 at 5hr post-drop to 4.4±1.1 ($p<0.0393$) at 2W post-drop, while High-C was significantly lower at 5hr post-drop compared to 5.7±0.8 ($p<0.0316$) at pre-drop and 10.0±1.3 ($p<0.0002$) at 2W post-drop (Appendix A, table A.9).

*Chloride [$Cl^-$] urine-plasma fractional excretion (FE) rate (%)*

Mean chloride FE rate at pre-drop was significantly different between the experimental low salt diet group High-A at 16.0±4.9 compared to the control low salt diet group Control-A at 3.6±0.7 ($p<0.0043$), while at 5hr post-drop, the experimental high salt diet group High-C was 3.9±1.4 and were significantly lower than the control high salt diet group Control-C at 15.8±7.3 ($p<0.0068$) (Table 3). Additionally, at 5hr post-drop, Control-C was significantly higher than Control-A at 4.1±0.6 ($p<0.0082$) and the control medium salt diet group Control-B at 3.0±0.7 ($p<0.0028$) (Table 3).

The mean chloride FE rate changed between sampling times, with Control-A significantly decreasing from 5hr post-drop to 2.6±1.1 ($p<0.0314$) at 2W post-drop, while Control-C increased significantly from 3.5±0.3 at pre-drop to 5hr post-drop ($p<0.0002$) and to 6.5±0.6 ($p<0.0084$) at 2W post-drop (Appendix A, table A.9). Moreover, High-C was the only experimental group that changed significantly, having increased from 5hr post-drop to 9.2±1.4 ($p<0.0008$, Appendix A, table A.9) at 2W post-drop.

*Potassium [$K^+$] urine-plasma fractional excretion (FE) rate (%)*

The mean potassium FE rate was only significantly different between experimental and control groups at pre-drop, with the experimental low salt diet group High-A being 827.5±285.0 and significantly higher than the control low salt diet group Control-A at 28.7±7.5 ($p<0.0019$), while the experimental medium salt diet group High-B was 775.7±496.4 compared to the control

medium salt diet group Control-B at 42.5±9.6 ($p<0.0059$) (Table 3). Additionally, the experimental high salt diet group High-C was 50.9±11.0 and were significantly lower than both High-A ($p<0.0028$) and High-B ($p<0.0037$) (Table 3).

For control groups, only Control-A had a significant change in mean potassium FE rate between sampling times, decreasing from 49.2±12.6 at 5hr post-drop to 14.5±4.1 ($p<0.0021$) at 2W post-drop (Appendix A, table A.9). For experimental groups, High-A significantly decreased from pre-drop to 30.3±11.4 ($p<0.0173$) at 2W post-drop, while High-C was significantly lower at 29.7±12.7 for 5hr post-drop compared to pre-drop ($p<0.0299$) and 59.6±6.7 ($p<0.0026$) at 2W post-drop (Appendix A, table A.9).

### *Magnesium [$Mg^{2+}$] urine-plasma fractional excretion (FE) rate (%)*

Experimental and control groups with high salt diets had a significant difference in mean magnesium FE rate 5hr and 2W post-drop, with the experimental group High-C at 9.9±5.5 for 5hr post-drop being significantly lower than the control group Control-C at 129.7±39.9 ($p<0.0156$), and High-C was 99.8±17.8 for the 2W post-drop and significantly lower than Control-C at 232.7±86.4 ($p<0.0036$) (Table 3). Additionally, at 5hr post-drop, Control-C was significantly higher than the control low salt diet group Control-A at 22.7±7.1 ($p<0.0366$) and the control medium salt diet group Control-B at 17.6±5.1 ($p<0.0369$) (Table 3). Also, for the 2W post-drop, Control-C was significantly higher than Control-A at 39.9±14.1 ($p<0.0001$) and Control-B at 26.2±5.3 ($p<0.0001$) (Table 3).

For control groups, the mean magnesium FE rate changed significantly between sampling times, with Control-B increasing from 12.6±2.5 at pre-drop to 2W post-drop ($p<0.0207$), and Control-C having increased significantly from 23.0±3.8 at pre-drop to 5hr post-drop ($p<0.0212$) and 2W post-drop ($p<0.0033$) (Appendix A, table A.9). For experimental groups, the mean magnesium FE rate significantly changed for the experimental medium salt diet group High-B having increased significantly to 62.7±20.1 at 2W post-drop from 16.6±4.8 ($p<0.0483$) at pre-drop and from 15.7±4.6 ($p<0.0152$) at 5hr post-drop, while High-C significantly decreased from 20.6±5.6 at pre-drop to 5hr post-drop ($p<0.0231$) and then increased to 2W post-drop (Pre-drop: $p<0.0024$, 5hr: $p<0.0001$) (Appendix A, table A.9).

### *Calcium [$Ca^{2+}$] urine-plasma fractional excretion (FE) rate (%)*

The mean calcium FE rate was only different between groups at 2W post-drop, with the experimental high salt diet group High-C at 232.5±83.4 being significantly higher than the control high salt diet group Control-C at 86.1±21.0 ($p<0.0001$, Table 3). Additionally, High-C

was significantly higher the experimental low salt diet group High-A at 3.4±0.9 ($p<0.0001$) and the experimental medium salt diet group High-B at 29.5±13.8 ($p<0.0001$) (Table 3).

Between sampling times, the mean calcium FE rate for the control medium salt diet group Control-B had increased significantly to 48.2±20.6 at 2W post-drop from 2.2±0.4 ($p<0.0064$) at pre-drop and from 2.4±0.8 ($p<0.0008$) at 5hr post-drop, while Control-C increased significantly from 2.5±0.5 at pre-drop to 9.6±3.2 ($p<0.0268$) at 5hr post-drop and then to 2W post-drop (Pre-drop: $p<0.0001$, 5hr: $p<0.0001$) (Appendix A, table A.9). For experimental groups, High-B had increased significantly to 2W post-drop from 1.9±0.5 ($p<0.0012$) at pre-drop and from 3.0±1.1 ($p<0.0022$) at 5hr post-drop, while High-C had significantly increased to 2W post-drop from 1.2±0.2 ($p<0.0001$) at pre-drop and from 1.6±0.7 ($p<0.0001$) at 5hr post-drop (Appendix A, table A.9).

### *Phosphate [$PO_4^{3-}$] urine-plasma fractional excretion (FE) rate (%)*

The mean phosphate FE rate was generally higher for control groups compared to the experimental groups, but no significant differences were observed (Table 3). For the 2W post-drop, the control high salt diet group Control-C at 111.8±14.4 was significantly higher than the control low salt diet group Control-A at 36.6±22.9 ($p<0.0015$, Table 3). Additionally, for experimental groups at 2W post-drop, the experimental high salt diet group High-C at 92.5±12.2 was significantly higher than both the experimental low salt diet group High-A at 12.6±4.2 ($p<0.0004$) and the experimental medium salt diet group High-B at 22.8±9.5 ($p<0.0032$) (Table 3).

Between sampling times, the mean calcium FE rate significantly changed for control groups, with the control medium salt diet group Control-B significantly increased from 28.7±9.6 at 5hr post-drop to 75.1±15.5 ($p<0.0408$) at 2W post-drop, while Control-C increased significantly at 2W post-drop from 20.7±6.7 ($p<0.0001$) at pre-drop and from 43.1±22.5 ($p<0.0008$) at 5hr post-drop (Appendix A, table A.9). For experimental groups, only High-C significantly changed between sampling times, going from 12.5±2.4 at pre-drop and significantly decreasing to 5.9±2.0 ($p<0.0158$) at 5hr post-drop and then increased to 2W post-drop (Pre-drop: $p<0.0001$, 5hr: $p<0.0001$) (Appendix A, table A.9).

### *Bicarbonate [$HCO_3^-$] urine-plasma fractional excretion (FE) rate (%)*

There were no significant differences in the mean bicarbonate FE rate between groups (Table 3). However, the mean bicarbonate FE rate for the control high salt diet group Control-C significantly decreased to 1.4±0.4 at 2W post-drop from 54.2±21.3 ($p<0.0001$) at pre-drop and

31.5±10.7 ($p<0.0001$) at 5hr post-drop (Appendix A, table A.9). For experimental groups, the low salt diet group High-A was significantly lower at 6.9±1.6 for 5hr post-drop compared to 35.4±10.8 ($p<0.0014$) at pre-drop and 30.0±6.5 ($p<0.0007$) at 2W post-drop, the medium salt diet group High-B had significantly decreased to 13.1±5.8 at 2W post-drop from 89.7±27.5 ($p<0.0001$) at pre-drop and 35.7±10.7 ($p<0.0056$) at 5hr post-drop, while the high salt diet group High-C significantly decreased to 2.0±0.8 at 2W post-drop from 316.0±268.5 ($p<0.0001$) at pre-drop and 37.2±19.2 ($p<0.0058$) at 5hr post-drop (Appendix A, table A.9).

*Partial pressure $CO_2$ ($PCO_2$) urine-plasma fractional excretion (FE) rate (%)*

The mean $PCO_2$ FE rate was significantly different between experimental and control groups for the low salt diets at pre-drop and the high salt diets at 5hr post-drop, with the experimental low salt diet group High-A being 18.4±10.7 at pre-drop and significantly higher than the control low salt diet group Control-A at 2.7±0.8 ($p<0.0091$), while the experimental high salt diet group High-C was 3.1±1.4 at 5hr post-drop and significantly lower than the control high salt diet group Control-C at 17.4±7.1 ($p<0.0147$) (Table 3). Additionally, for experimental groups at pre-drop, High-A was significantly higher than both the medium salt diet group High-B at 3.0±0.7 ($p<0.0107$) and High-C at 4.1±0.8 ($p<0.0235$) (Table 3). For control groups at 5hr post-drop, Control-C was significantly higher than both the low salt diet group Control-A at 1.7±0.4 ($p<0.0067$) and the medium salt diet group Control-B at 2.0±0.6 ($p<0.0064$) (Table 3).

For control groups between sampling times, Control-A had a significant increase in mean $PCO_2$ FE rate to 9.6±5.2 at 2W post-drop from pre-drop ($p<0.0369$) and 5hr post-drop ($p<0.0113$), while Control-C increased significantly from 2.3±0.4 at pre-drop to 5hr post-drop ($p<0.0022$) and to 4.6±1.7 ($p<0.0051$) at 2W post-drop (Appendix A, table A.9). For experimental groups, only High-C significantly changed between sampling times, significantly decreasing from pre-drop to 1.3±0.3 ($p<0.0186$) at 2W post-drop (Appendix A, table A.9).

*Total $CO_2$ ($TCO_2$) urine-plasma fractional excretion (FE) rate (%)*

For the 5hr post-drop, the control high salt diet group Control-C was 36.5±12.7 and had significantly higher mean $TCO_2$ FE rate compared to the experimental high salt diet group High-C at 5.4±1.8 ($p<0.0001$, Table 3). Additionally, Control-C was significantly higher than the control low salt diet group Control-A at 3.0±0.8 ($p<0.0001$) and the control medium salt diet group Control-B at 2.9±1.1 ($p<0.0001$) (Table 3).

The only control group with a significant change in mean $TCO_2$ FE rate was Control-C, which significantly increased from 5.6±1.1 at pre-drop to 5hr post-drop ($p<0.0004$), and then

significantly decreased to 2.1±0.5 ($p<0.0001$) at 2W post-drop (Appendix A, table A.9). For experimental groups, the medium salt diet group High-B significantly decreased to 4.1±1.4 at 2W post-drop from 7.5±1.5 ($p<0.0377$) at pre-drop and 8.1±2.2 ($p<0.0380$) at 5hr post-drop, while High-C was significantly higher at 10.0±1.5 for the pre-drop before decreasing to 5hr post-drop ($p<0.0232$) and 2.6±0.5 ($p<0.0036$) at 2W post-drop (Appendix A, table A.9).

### *Albumin urine-plasma fractional excretion (FE) rate (%)*

The mean albumin FE rate was significantly different between the experimental medium salt diet group High-B at 3.68±1.60 compared to the control medium salt diet group Control-B at 0.83±0.43 ($p<0.0007$, Table 3) at 5hr post-drop. Additionally, for experimental groups at 5hr post-drop, High-B was significantly higher than the low salt diet group High-A at 1.13±0.21 ($p<0.0025$) and the high salt diet group High-C at 0.12±0.04 ($p<0.0001$) (Table 3).

Only High-C had a significant change in mean albumin FE rate between sampling times, being significantly higher at 0.47±0.10 for pre-drop and then significantly decreasing to 5hr post-drop ($p<0.0020$) and 0.21±0.05 ($p<0.0278$) at 2W post-drop (Appendix A, table A.9).

**Table 3: Urine-plasma fractional excretion (FE) rate (%) for all ion and metabolite concentrations in juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups. 0% indicates complete reabsorption, 99% is very low reabsorption, and values of 100% or higher indicate complete excretion.** Letters indicate differences between sampling times within groups. Within the control $CO_2$ groups, blue asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while blue diamond (◆) indicates a significant difference between medium and high salt diet. within the control $CO_2$ group. Within high $CO_2$ groups, red asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while red diamond shape (◆) indicates a significant difference between medium and high salt diet. Pluss sign (+) indicates a significant difference between control and high $CO_2$ treatment within diet groups. Data is presented as mean and standard error of mean (SEM).

| Urine-Plasma fractional excretion (FE) rate (%) | | | | | | |
|---|---|---|---|---|---|---|
| Group / Time | Control-A | Control-B | Control-C | High-A | High-B | High-C |
| | | | [$Na^+$] | | | |
| Pre-drop | $4.8\pm0.8^{ab}$ | $5.3\pm0.8^{a}$ | $4.1\pm0.3^{a}$ | $5.7\pm1.4^{ab}$ | $4.2\pm0.8^{a}$ | $5.7\pm0.8^{a}$ |
| 5hr post-drop | $5.2\pm0.7^{a}$ | $3.8\pm0.8^{a}$ | $18.0\pm8.2^{b*◆+}$ | $8.4\pm0.7^{a}$ | $8.6\pm2.8^{a}$ | $3.8\pm1.3^{b+}$ |
| 2W post-drop | $3.6\pm1.5^{b}$ | $4.7\pm0.6^{a}$ | $7.5\pm0.9^{b}$ | $4.4\pm1.1^{b}$ | $3.1\pm0.7^{a}$ | $10.0\pm1.3^{a}$ |
| | | | [$Cl^-$] | | | |
| Pre-drop | $3.6\pm0.7^{ab+}$ | $3.8\pm0.7^{a}$ | $3.5\pm0.3^{a}$ | $16.0\pm4.9^{a+}$ | $7.9\pm3.6^{a}$ | $5.7\pm1.0^{ab}$ |
| 5hr post-drop | $4.1\pm0.6^{a}$ | $3.0\pm0.7^{a}$ | $15.8\pm7.3^{b*◆+}$ | $6.6\pm0.7^{a}$ | $7.3\pm2.3^{a}$ | $3.9\pm1.4^{a+}$ |
| 2W post-drop | $2.6\pm1.1^{b}$ | $3.2\pm0.5^{a}$ | $6.5\pm0.6^{b}$ | $3.6\pm0.9^{a}$ | $2.9\pm0.6^{a}$ | $9.2\pm1.4^{b}$ |
| | | | [$K^+$] | | | |
| Pre-drop | $28.7\pm7.5^{ab+}$ | $42.5\pm9.6^{ab+}$ | $53.1\pm18.2^{a}$ | $827.5\pm285.0^{a+}$ | $775.7\pm496.4^{a+}$ | $50.9\pm11.0^{a}$ |
| 5hr post-drop | $49.2\pm12.6^{a}$ | $26.1\pm7.2^{a}$ | $209.9\pm132.7^{a}$ | $58.4\pm9.2^{ab}$ | $38.1\pm9.3^{a}$ | $29.7\pm12.7^{b}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2W post-drop | 14.5±4.1[b] | 26.6±5.1[a] | 60.6±11.1[a] | 30.3±11.4[b] | 26.8±7.6[a] | 59.6±6.7[a] |
| [$Mg^{2+}$] | | | | | | |
| Pre-drop | 36.7±17.9[a] | 12.6±2.5[a] | 23.0±3.8[a] | 44.9±27.5[a] | 16.6±4.8[a] | 20.6±5.6[a] |
| 5hr post-drop | 22.7±7.1[a] | 17.6±5.1[ab] | 129.7±40.0[b*◆+] | 12.2±2.1[a] | 15.7±4.6[a] | 9.9±5.5[b+] |
| 2W post-drop | 39.9±14.1[a] | 26.2±5.3[b] | 232.7±86.4[b*◆+] | 11.1±3.9[a] | 62.7±20.1[b] | 99.8±17.8[c+] |
| [$Ca^{2+}$] | | | | | | |
| Pre-drop | 3.4±1.6[a] | 2.2±0.4[a] | 2.5±0.5[a] | 2.6±0.8[a] | 1.9±0.5[a] | 1.2±0.2[a] |
| 5hr post-drop | 2.7±0.6[a] | 2.4±0.8[a] | 9.6±3.2[b] | 1.2±0.2[a] | 3.0±1.1[a] | 1.6±0.7[a] |
| 2W post-drop | 3.6±1.1[a] | 48.2±20.6[b] | 86.1±21.0[c+] | 3.4±0.9[a] | 29.5±13.8[b] | 232.5±83.4[b*◆+] |
| [$PO_4^{3-}$] | | | | | | |
| Pre-drop | 34.9±14.9[a] | 33.9±8.1[ab] | 20.7±6.7[a] | 62.0±35.1[a] | 7.5±2.3[a] | 12.5±2.4[a] |
| 5hr post-drop | 35.7±12.8[a] | 28.7±9.6[a] | 43.1±22.5[a] | 4.8±1.3[a] | 8.6±2.6[a] | 5.9±2.0[b] |
| 2W post-drop | 36.6±22.9[a] | 75.1±15.5[b] | 111.8±14.4[b*] | 12.6±4.2[a] | 22.8±9.5[a] | 92.5±12.2[c*◆] |
| TCO2 | | | | | | |
| Pre-drop | 2.9±0.8[a] | 4.7±1.0[a] | 5.6±1.1[a] | 5.2±1.0[a] | 7.5±1.5[a] | 10.0±1.5[a] |
| 5hr post-drop | 3.0±0.8[a] | 2.9±1.1[a] | 36.5±12.7[b*◆+] | 4.2±0.9[a] | 8.1±2.2[a] | 5.4±1.8[b+] |
| 2W post-drop | 6.8±1.5[a] | 3.5±1.0[a] | 2.1±0.5[a] | 7.6±2.9[a] | 4.1±1.4[b] | 2.6±0.5[b] |
| PCO2 | | | | | | |
| Pre-drop | 2.7±0.8[a+] | 3.2±0.5[a] | 2.3±0.4[a] | 18.4±10.7[a+] | 3.0±0.7[a] | 4.1±0.8[a] |
| 5hr post-drop | 1.7±0.4[a] | 2.0±0.6[a] | 17.4±7.1[b*◆+] | 2.6±0.4[a] | 3.5±1.0[a] | 3.1±1.4[ab] |
| 2W post-drop | 9.6±5.2[b] | 4.2±1.3[a] | 4.6±1.7[b] | 3.7±1.3[a] | 3.0±1.1[a] | 1.3±0.3[b] |
| [$HCO_3^-$] | | | | | | |
| Pre-drop | 87.8±50.1[a] | 106.4±80.3[a] | 54.2±21.3[a] | 35.4±10.8[a] | 89.7±27.5[a] | 316.0±268.5[a] |
| 5hr post-drop | 144.4±6.6[a] | 22.0±5.4[a] | 31.5±10.7[a] | 6.9±1.6[b] | 35.7±10.7[a] | 37.2±19.2[a] |
| 2W post-drop | 52.7±26.1[a] | 80.8±63.3[a] | 1.4±0.4[b] | 30.0±6.5[a] | 13.1±5.8[b] | 2.0±0.8[b] |
| Albumin | | | | | | |
| Pre-drop | 1.4±0.48[a] | 0.6±0.21[a] | 0.1±0.04[a] | 0.6±0.21[a] | 0.2±0.06[a] | 0.5±0.10[a] |
| 5hr post-drop | 1.3±0.28[a] | 0.8±0.43[a+] | 0.4±0.27[a] | 1.1±0.21[a+] | 3.7±1.60[a] | 0.1±0.04[b] |
| 2W post-drop | 0.4±0.14[a] | 0.6±0.14[a] | 1.2±0.46[a] | 1.2±0.65[a] | 0.5±0.18[a] | 0.2±0.05[b] |

### 3.1.9 $Na^+/K^+$ ATPase (NKA) enzyme activity

*Proximal intestine NKA enzyme activity (µmol ADP/hr/mg protein)*

The NKA activity in the proximal intestine was significantly different between control and experimental groups, where the control group Control-C was 20.2±2.3 µmol ADP/hr/mg protein compared to the experimental group High-C at 10.8±1.6 µmol ADP/hr/mg protein ($p<0.0005$) at 5hr post-drop, while High-C was 35.0±3.3 µmol ADP/hr/mg protein and higher than Control-C at 27.4±2.3 µmol ADP/hr/mg protein ($p<0.0104$) for the 2W post-drop (Fig. 16a).

For control groups, Control-C was significantly higher than both the low salt diet group Control-A at 1.0±0.4 µmol ADP/hr/mg protein ($p<0.0001$) and the medium salt diet group Control-B at 6.6±2.0 µmol ADP/hr/mg protein ($p<0.0001$) for the 5hr post-drop, while Control-C was significantly higher than Control-A at 16.8±1.3 µmol ADP/hr/mg protein ($p<0.0001$) for the 2W post-drop (Fig. 16.a). For experimental groups, High-C was significantly higher than High-A for both 1.1±0.4 µmol ADP/hr/mg protein ($p<0.0005$) at 5hr post-drop and 17.5±1.4 µmol ADP/hr/mg protein ($p<0.0001$) at 2W post-drop (Fig. 16a).

Between sampling times, all groups had significantly increased NKA enzyme activity in the proximal intestine. For control groups, Control-A started at 3.2±0.9 µmol ADP/hr/mg protein pre-drop and significantly decreased to 5hr post-drop ($p<0.0049$) before increasing to 2W post-drop (Pre-drop: $p<0.0001$, 5hr: $p<0.0001$), the medium salt diet group Control-B started at 5.1±1.0 µmol ADP/hr/mg protein pre-drop and significantly increased to 6.6±2.0 µmol ADP/hr/mg protein ($p<0.0001$) at 5hr post-drop prior to increasing further to 23.2±1.6 µmol ADP/hr/mg protein (Pre-drop: $p<0.0145$, 5hr: $p<0.0001$), while Control-C increased significantly at 2W post-drop from 4.3±0.6 µmol ADP/hr/mg protein ($p<0.0003$) at pre-drop and 5hr post-drop ($p<0.0001$) (Appendix A, table A.1). For experimental groups, High-A increased at 2W post-drop from 4.9±0.7 µmol ADP/hr/mg protein ($p<0.0007$) at pre-drop and from 5hr post-drop ($p<0.0001$), the medium salt diet group High-B started at 5.5±0.5 µmol ADP/hr/mg protein at pre-drop and decreased to 4.6±1.6 µmol ADP/hr/mg protein ($p<0.0001$) at 5hr post-drop before increasing to 19.6±2.2 µmol ADP/hr/mg protein (Pre-drop: $p<0.0001$, 5hr: 0.0337) at 2W post-drop, while High-C had increased at 2W post-drop from 2.3±0.4 µmol ADP/hr/mg protein ($p<0.0001$) at pre-drop and from 5hr post-drop ($p<0.0097$) (Appendix A, table A.1).

*Distal intestine NKA enzyme activity (µmol ADP/hr/mg protein)*

The mean NKA enzyme activity in the distal intestine was not significantly different between experimental and control groups. For control groups at 5hr post-drop, the high salt diet group Control-C at 4.17±0.74 µmol ADP/hr/mg protein was significantly higher than the low salt diet group Control-A at 0.31±0.07 µmol ADP/hr/mg protein ($p<0.0002$, Fig. 15b). Additionally, for 2W post-drop, Control-C was 4.92±0.96 µmol ADP/hr/mg protein compared to Control-A at 1.04±0.40 µmol ADP/hr/mg protein ($p<0.0002$, Fig. 15b). For experimental groups at 2W post-drop, the high salt diet group High-C at 6.08±0.8 µmol ADP/hr/mg protein was significantly higher than both the low salt diet group High-A at 3.49±0.59 µmol ADP/hr/mg protein ($p<0.0356$) and the medium salt diet group High-B at 4.92±0.96 µmol ADP/hr/mg protein ($p<0.0012$) (Fig. 15b).

The mean NKA enzyme activity changed over time, with Control-A decreasing from 2.80±1.50 µmol ADP/hr/mg protein at pre-drop to 5hr post-drop ($p<0.0091$), the control group with medium salt diet Control-B increased significantly to 3.13±0.61 µmol ADP/hr/mg protein at 2W post-drop from 1.16±0.28 µmol ADP/hr/mg protein ($p<0.0283$) at pre-drop and 1.90±0.9 µmol ADP/hr/mg protein ($p<0.0416$) at 5hr post-drop, while Control-C started at 0.68±0.09 µmol ADP/hr/mg protein pre-drop and increased significantly to 5hr post-drop ($p<0.0001$) and 2W post-drop ($p<0.0001$) (Appendix A, table A.1). For experimental groups, High-A increased significantly at 2W post-drop from 0.38±0.08 µmol ADP/hr/mg protein ($p<0.0005$) at pre-drop and 5hr post-drop µmol ADP/hr/mg protein ($p<0.0001$), High-B increased to 2.61±0.47 at 2W post-drop from 0.78±0.26 µmol ADP/hr/mg protein ($p<0.0482$) at pre-drop and 0.74±0.41 µmol ADP/hr/mg protein ($p<0.0018$) at 5hr post-drop, while High-C increased significantly to 2W post-drop from 0.80±0.12 µmol ADP/hr/mg protein ($p<0.0001$) at pre-drop and 1.73±0.19 µmol ADP/hr/mg protein ($p<0.0215$) at 5hr post-drop (Appendix A, table A.1).

*Gills NKA enzyme activity (µmol ADP/hr/mg protein)*

The mean NKA enzyme activity in the gills was significantly different between experimental and control groups at pre-drop, where the control group with low salt diet Control-A was 11.0±0.4 µmol ADP/hr/mg protein and being significantly higher than the respective experimental group High-A at 8.2±0.6 µmol ADP/hr/mg protein ($p<0.0384$), while the control group with high salt diet Control-C was 10.7±0.4 µmol ADP/hr/mg protein and significantly higher than the experimental group High-C at 7.8±0.9 µmol ADP/hr/mg protein ($p<0.0234$) (Fig. 15c).

At 5hr post-drop, Control-C was 5.8±1.0 µmol ADP/hr/mg protein and had significantly lower NKA enzyme activity compared to both Control-A at 8.7±0.6 µmol ADP/hr/mg protein ($p<0.0313$) and the medium salt diet group Control-B at 10.2±0.6 µmol ADP/hr/mg protein ($p<0.0001$) (Fig. 15c).

For control groups, the NKA enzyme activity changed over time for the low and high salt diets, with Control-A significantly decreasing from pre-drop to 5hr post-drop ($p<0.0183$), and Control-C significantly decreasing from pre-drop to 5hr post-drop ($p<0.0001$) and then increasing to 9.8±0.6 µmol ADP/hr/mg protein at 2W post-drop ($p<0.0006$) (Appendix A, table A.1). For experimental groups, the medium salt diet group High-B increased significantly to 11.9±0.5 µmol ADP/hr/mg protein at 2W post-drop from 9.3±0.8 µmol ADP/hr/mg protein ($p<0.0350$) at pre-drop and 9.0±0.7 µmol ADP/hr/mg protein ($p<0.0126$) at 5hr post-drop, while Control-C increased significantly from 7.7±0.6 µmol ADP/hr/mg protein at 5hr post-drop to 9.7±0.6 µmol ADP/hr/mg protein ($p<0.0394$) at 2W post-drop (Appendix A, table A.1).

### *Kidney NKA enzyme activity (µmol ADP/hr/mg protein)*

The only significant differences in mean kidney NKA enzyme activity between groups was for experimental groups at pre-drop, where the medium salt diet group High-B at 16.7±1.1 µmol ADP/hr/mg protein was significantly higher than the low salt diet group High-A at 12.4±0.8 µmol ADP/hr/mg protein ($p<0.0212$) and the high salt diet group High-A at 11.6±1.5 µmol ADP/hr/mg protein ($p<0.0031$) (Fig. 15d).

For control groups, the kidney NKA enzyme activity only significantly changed for the medium salt diet group Control-B, which decreased from 15.0±0.8 µmol ADP/hr/mg protein at pre-drop to 12.1±0.9 µmol ADP/hr/mg protein ($p<0.0373$, Appendix A, table A.1) at 5hr post-drop. For experimental groups, the low and medium salt diet groups significantly changed between sampling times, with High-A having increased to 15.8±1.0 µmol ADP/hr/mg protein at 2W post-drop from pre-drop ($p<0.0080$) and 13.1±0.6 µmol ADP/hr/mg protein ($p<0.0416$) at 5hr post-drop, while High-B decreased from pre-drop to 11.7±0.7 µmol ADP/hr/mg protein ($p<0.0032$) at 5hr post-drop and then increased to 16.3±1.1 µmol ADP/hr/mg protein ($p<0.0066$) at 2W post-drop (Appendix A, table A.1).

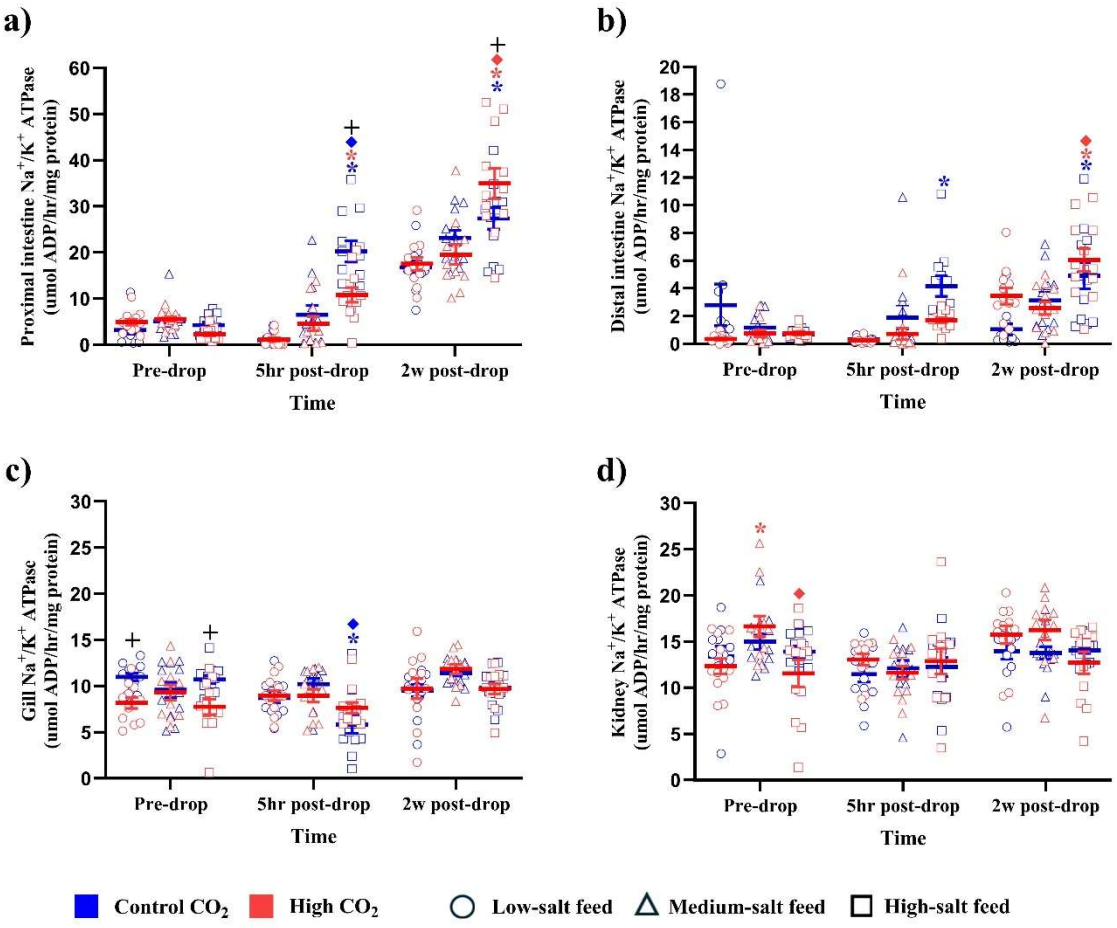


**Figure 15: $Na^+/K^+$ ATPase enzyme activity in osmoregulatory organs of juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Within the control $CO_2$ groups, blue asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while blue diamond (◆) indicates a significant difference between medium and high salt diet. within the control $CO_2$ group. Within high $CO_2$ groups, red asterisk (*) indicates a significant difference for high or medium salt diets compared to the low salt diet, while red diamond shape (◆) indicates a significant difference between medium and high salt diet. Pluss sign (+) indicates a significant difference between control and high $CO_2$ treatment within diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

### 3.1.10 Hematology

*Hematocrit (Hct) %*

The only significant difference for mean hematocrit percentage in blood was for the high salt diet groups at 5hr post-drop, where the control group Control-C was 37.3±1.0 % and were significantly higher than the experimental group High-C at 32.4±0.9 % ($p<0.0436$, Fig. 16a).

Between sampling times, the hematocrit percentage significantly changed for the experimental groups with the low and high salt diets, with the low salt diet group High-A decreasing from 37.8±3.4 % at pre-drop to 31.3±1.0 % ($p<0.0363$) at 2W post-drop, while High-C decreased from 34.8±0.8 % at pre-drop to 5hr post-drop ($p<0.0187$) (Appendix A, table A.2).

*Drabkin hemoglobin concentration (g/dL)*

No significant differences were found between groups within sampling times (Fig. 16b), and there were no groups that changed significantly between sampling times (Appendix A, table A.2).

*Drabkin Mean Corpuscular Hemoglobin Concentration (MCHC) (g/dL)*

There were no significant differences found between groups within sampling times (Fig. 16c).

The Drabkin MCHC increased significantly between sampling times for the control groups with medium and high salt diets, with the medium salt diet group Control-B rising from 21.7±1.3 g/dL at 5hr post-drop to 26.0±1.1 g/dL ($p<0.0371$) at 2W post-drop, while high salt diet group Control-C going from 22.9±1.7 at 5hr post-drop to 27.8±0.8 g/dL ($p<0.0194$) at 2W post-drop (Appendix A, table A.2).

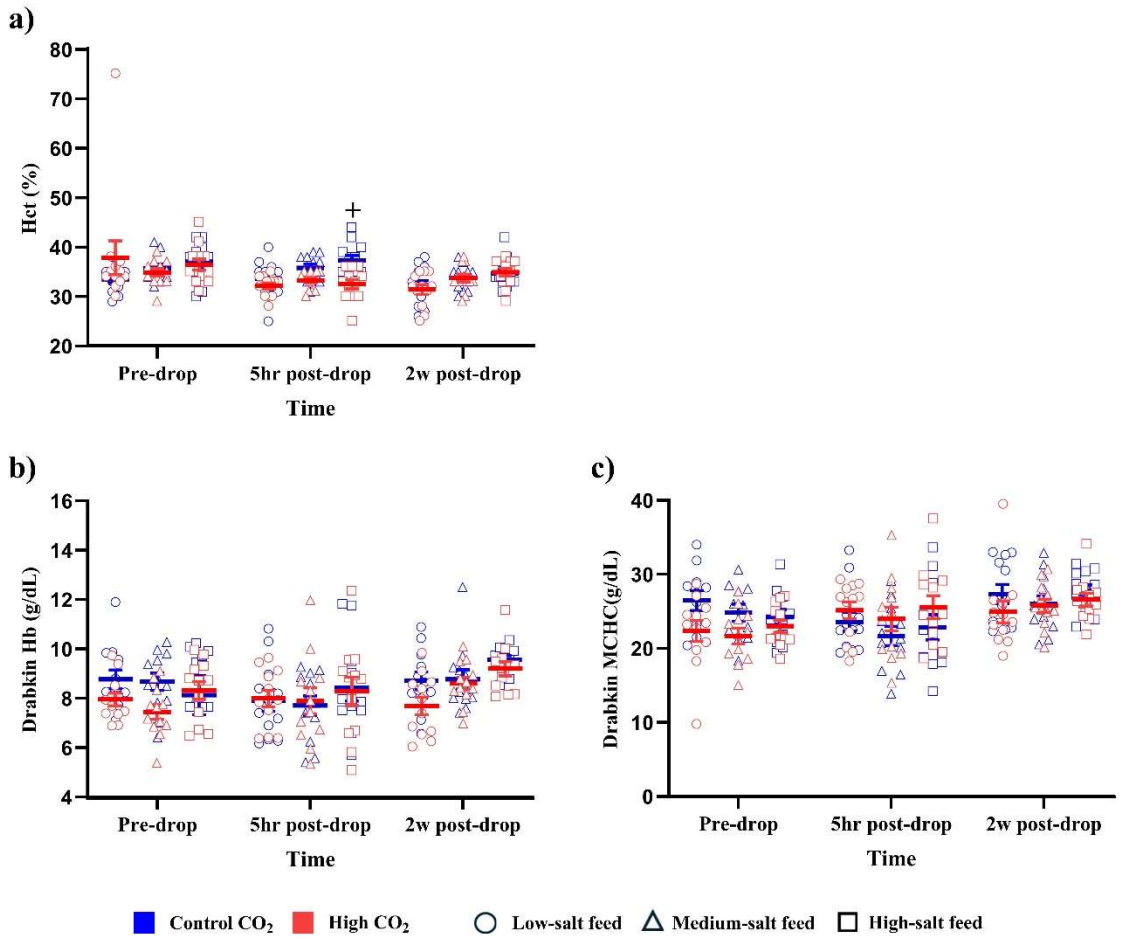


**Figure 16: Hematological parameter concentrations in blood samples from juvenile Atlantic salmon in freshwater (FW) pre-, 5hr post- and 2W post-drop of water level $CO_2$ from high (30 mg/L) to control (<8 mg/L) for high $CO_2$ groups.** Pluss sign (+) indicates a significant difference between control and high $CO_2$ treatment within diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

# 3.2 Urinary catheterization trial

## 3.2.1 Animal performance

### *Body mass (g)*

The mean body mass of the experimental groups was significantly lower than their respective control group, with the control low salt diet group Control-A at 930.8±41.7 g compared to the experimental low salt diet group High-A at 706.8±30.0 g ($p<0.0007$), the control medium salt diet group Control-B was 935.8±36.3 g compared to the experimental medium salt diet group High-B at 708.3±52.3 g ($p<0.0003$), while the control high salt diet group Control-C was 909.5±46.9 g compared to the experimental high salt diet group High-C at 670.6±39.6 g ($p<0.0006$) (Fig. 17a).

*Specific growth rate (SGR) %*

The mean SGR was significantly different between experimental and control groups with low and high salt diets, with the control low salt diet group Control-A at 96.4±5.0 % and significantly higher than the experimental low salt diet group High-A was 79.4±2.9 % ($p<0.0012$), while the control high salt diet group Control-C was 84.1±2.8 % and significantly higher than the experimental high salt diet group High-C at 72.3±4.0 % ($p<0.0388$) (Fig. 18b). Additionally, between control groups, the medium salt diet group Control-B at 82.4±4.8 % was significantly lower than Control-A ($p<0.0334$, Fig. 17b).

*Condition factor (CF)*

The mean CF was significantly different between experimental and respective control groups, with the control low salt diet group Control-A at 1.32±0.04 compared to the experimental low salt diet group High-A at 1.16±0.03 ($p<0.0010$), the control medium salt diet group Control-B was 1.25±0.04 compared to the experimental medium salt diet group High-B at 1.14±0.03 ($p<0.0188$), while the control high salt diet group Control-C was 1.24±0.04 compared to the experimental high salt diet group High-C at 1.10±0.03 ($p<0.0112$) (Fig. 17c).

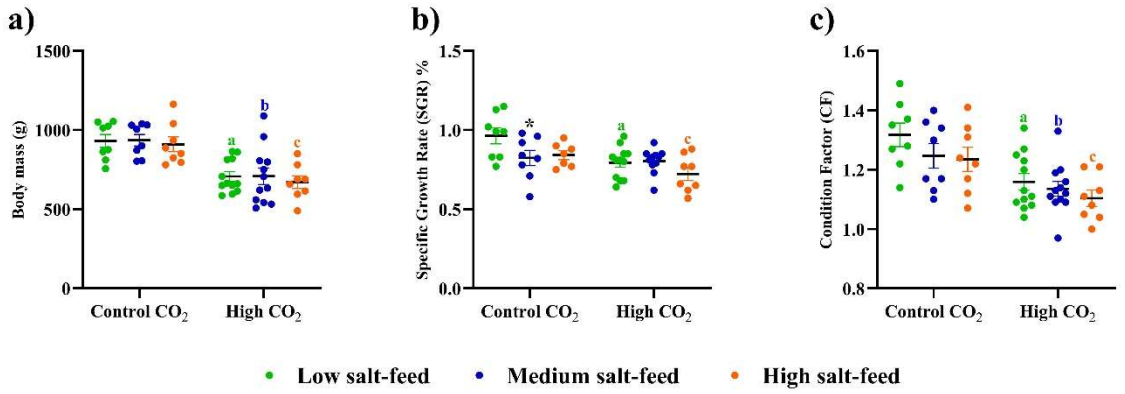


**Figure 17: Body mass (g), specific growth rate (SGR) and condition factor (CF) of juvenile Atlantic salmon in freshwater (FW) after 72 hours of catheterization and 24 hours post-drop of water level $CO_2$, from high (30 mg/L) for experimental groups, and control (<8 mg/L) for control groups, to almost 0 mg/L for all groups.** Letters indicate significant differences within each diet group. An asterisk (*) indicates a significant difference between low and medium salt diet groups within $CO_2$ treatment. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

### 3.2.2 Urine 48-hour pH and UFR changes

*Urine pH for high (experimental) $CO_2$ groups*

The mean urine pH of the low salt diet group High-A was 7.49±0.18 at P1 but was not significantly different before dropping water level $CO_2$ (Fig. 18a). Compared to P1, a significant rise in mean urine pH was observed at D4 with a value of 8.04±0.07 ($p<0.0339$) and the following sampling times D8 at 8.07±0.08 ($p<0.0185$) and D12 at 8.02±0.06 ($p<0.0477$) (Fig. 18a). The medium salt diet group High-B started at 7.29±0.07 for P1 and were significantly higher post-drop for D2 at 7.86±0.07 ($p<0.0390$), D4 at 7.93±0.06 ($p<0.0098$), D6 at 7.99±0.04 ($p<0.0024$), D8 at 8.03±0.06 ($p<0.0008$), D12 at 8.01±0.03 ($p<0.0015$), and D24 at 7.93±0.04 ($p<0.0084$) compared to P1(Fig 18a). The high salt diet group High-C started at 7.58±0.21 for P1 and was only significantly higher at D12 with a mean urine pH of 8.11±0.06 ($p<0.0347$, Fig. 18a).

No significant differences in urine pH were observed between groups during the trial (Fig. 18a).

*Urine pH for control $CO_2$ groups*

The mean urine pH of the low salt diet group Control-A was 7.37±0.11 at P1 and were significantly higher post-drop for D6 at 7.71±0.03 ($p<0.0160$), D8 at 7.86±0.05 ($p<0.0001$), D12 at 7.75±0.07 ($p<0.0027$) and D24 at 7.79±0.01 ($p<0.0005$) (Fig. 18b). The medium salt diet group Control-B started at 7.55±0.05 for P1 and was only significantly different post-drop for D8 at 7.92±0.05 ($p<0.0050$, Fig. 18b). The high salt diet group Control-C was 7.30±0.04 for P1 and were significantly higher pre-drop for P12 at 7.70±0.05 ($p<0.0103$), and post-drop for D2 at 7.88±0.05 ($p<0.0001$), D4 at 7.76±0.08 ($p<0.0007$), D6 at 7.72±0.05 ($p<0.0030$), D8 at 7.79±0.05 ($p<0.0002$), D12 at 7.65±0.05 ($p<0.0460$) and D24 at 7.68±0.03 ($p<0.0163$) (Fig. 18b).

The mean urine pH varied between groups at different sampling times pre-drop with Control-C being significantly lower than Control-B ($p<0.0351$) at P1, Control-B at 7.55±0.09 was significantly higher than Control-A at 7.27±0.07 ($p<0.0052$) for P2, Control-A at 7.47±0.07 was significantly lower than both Control-B at 7.74±0.08 ($p<0.0084$) and Control-C ($p<0.0366$) for P12, and Control-B at 7.67±0.07 was significantly higher than Control-A at 7.32±0.07 ($p<0.0002$) and Control-C at 7.40±0.09 ($p<0.0088$) for P24 (Fig. 18b). Groups was only significantly different post-drop for D2 with Control-C being significantly higher than Control-A at 7.61±0.07 ($p<0.0091$) and Control-B at 7.65±0.02 ($p<0.0379$, Fig. 18b).

*Urine flow rate (UFR) for high (experimental) $CO_2$ groups (mL/hr/kg)*

The UFR did not significantly change for any of the groups pre- or post-drop during 48 hours of sampling catheterized fish (Fig. 18c). The low salt diet group High-A had a generally higher UFR than the medium salt diet group High-B and the high salt diet group High-C, but was only significantly higher for P6 with High-A at 3.93±0.34 mL/hr/kg compared to High-C at 2.76±0.27 mL/hr/kg ($p<0.0139$), and for D8 with High-A at 3.61±0.08 mL/hr/kg compared to High-C at 3.20±0.40 mL/hr/kg ($p<0.0393$) (Fig. 18c).

*Urine flow rate (UFR) for control $CO_2$ groups (mL/hr/kg)*

The mean UFR for the low salt diet group Control-A and the high salt diet group Control-C did not significantly change between sampling times, but the medium salt diet group Control-B started at 2.93±0.19 mL/hr/kg for P1 and was significantly higher for D6 at 3.95±0.20 mL/hr/kg ($p<0.0432$, Fig. 18d).

Between groups, Control-A was 3.13±0.24 mL/hr/kg for D1 and were significantly higher than Control-B at 2.41±0.16 mL/hr/kg ($p<0.0388$) and Control-C at 2.24±0.13 mL/hr/kg ($p<0.0077$), while Control-C was 3.92±0.19 mL/hr/kg for D2 and were significantly higher than Control-B at 3.20±0.14 mL/hr/kg ($p<0.0404$) (Fig. 18c).

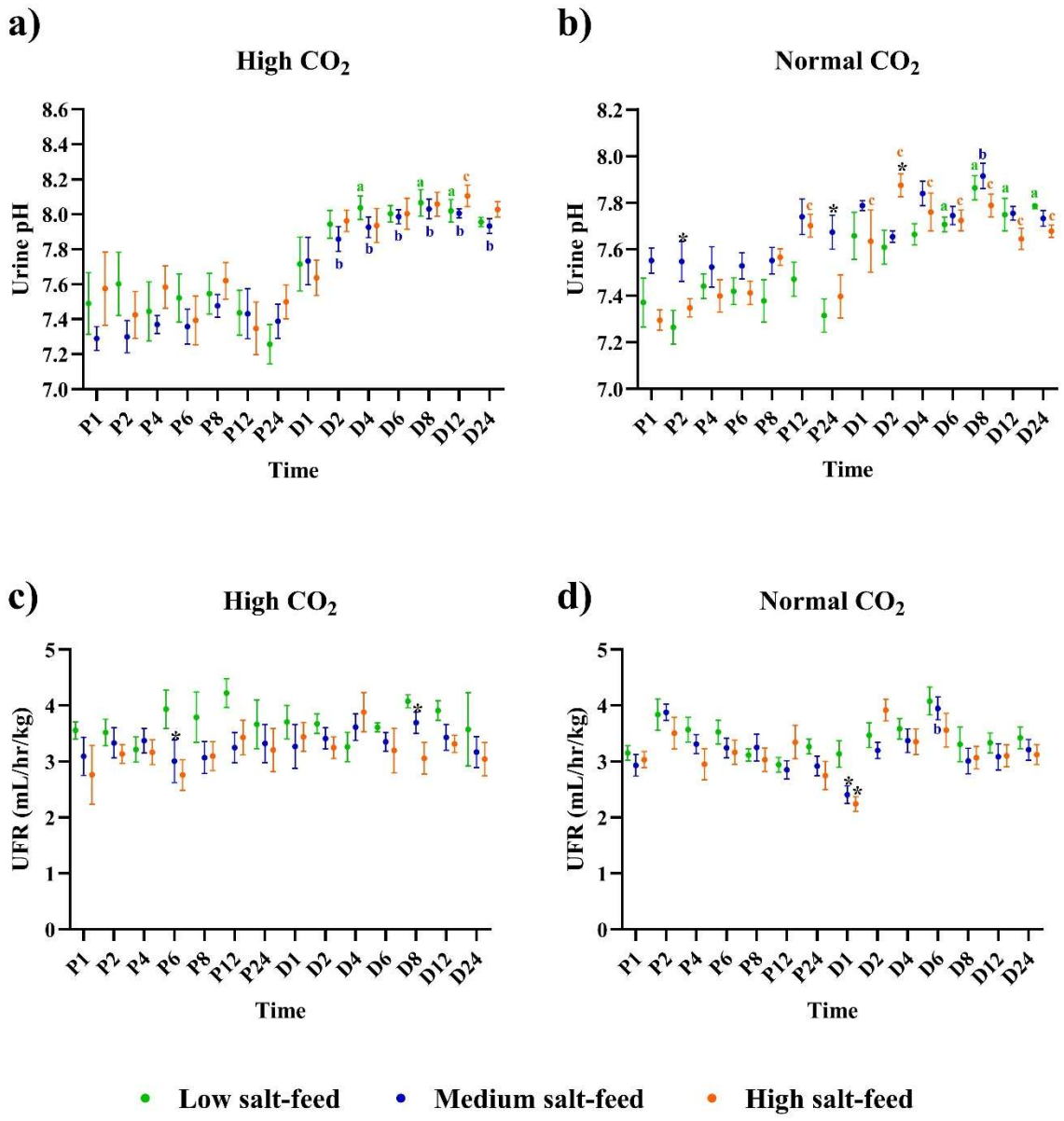


**Figure 18: Urine pH and urine flow rate (UFR) of juvenile Atlantic salmon in freshwater (FW) during 48 hours of catheterization, dropping water level $CO_2$ after 24 hours from high $CO_2$ (30 mg/L) for experimental groups and control $CO_2$ (<8 mg/L) for control groups, to almost 0 mg/L for all groups.** Letters indicate significant differences within each diet group. An asterisk (*) indicates a significant difference between low and medium salt diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

### 3.2.3 Na+/K+ ATPase enzyme activity

*Proximal intestine NKA enzyme activity (µmol ADP/hr/mg protein)*

For mean NKA enzyme activity, no significance was observed for diets or $CO_2$ treatments in proximal intestine (Fig. 19a).

*Distal intestine NKA enzyme activity (µmol ADP/hr/mg protein)*

There was no significant difference observed between groups for the mean NKA enzyme activity in the distal intestine (Fig. 19b).

*Gill NKA enzyme activity (µmol ADP/hr/mg protein)*

The mean NKA enzyme activity in gill tissue was significantly different between experimental and control $CO_2$ treatments for the high salt diet groups, from 3.7±1.0 in the experimental group High-C, to 4.9±1.4 ($p<0.0118$, Fig. 19c) for the control group Control-C.

*Kidney NKA enzyme activity (µmol ADP/hr/mg protein)*

No significant differences in mean kidney NKA enzyme activity were found between groups (Fig. 19d).

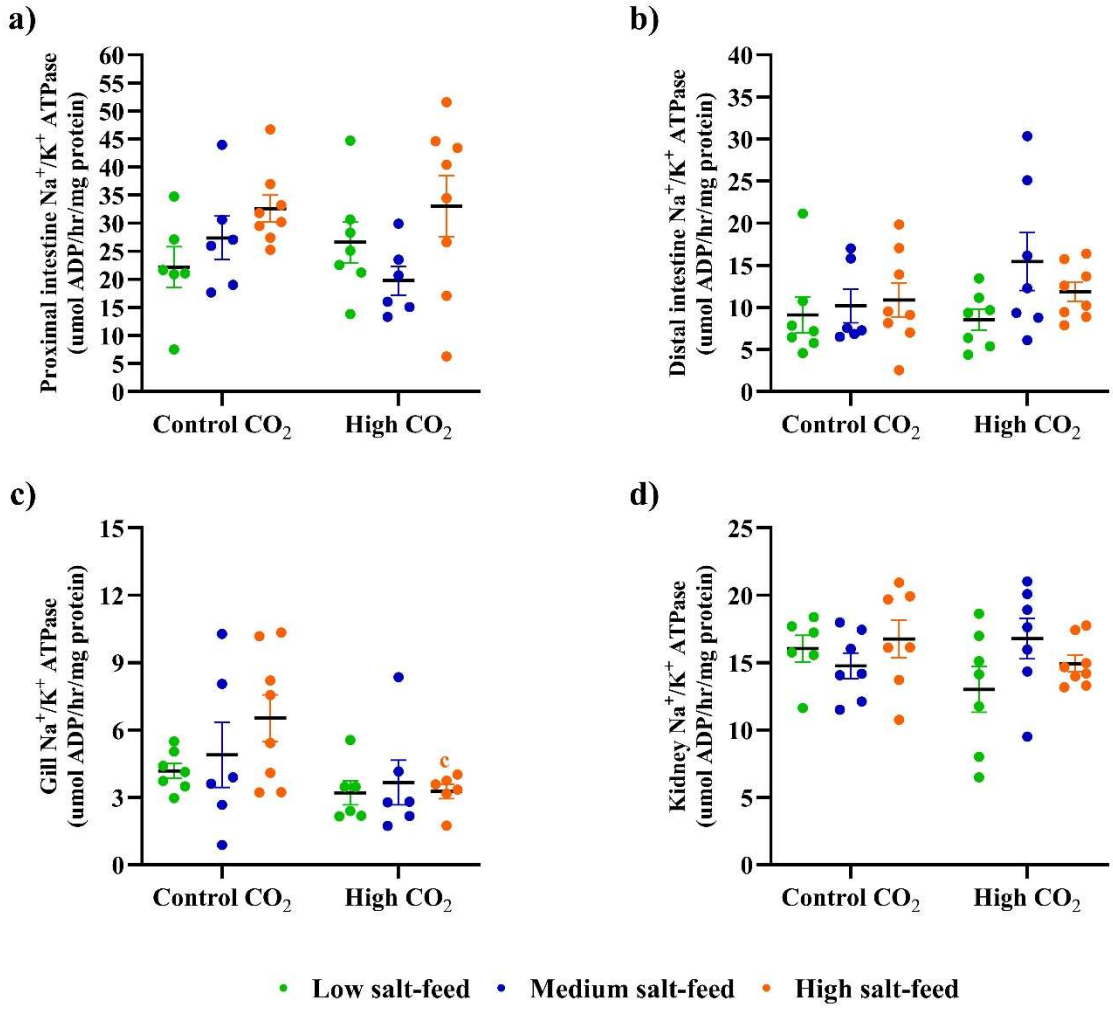


**Figure 19: The Na+/K+ ATPase enzyme activity in the main osmoregulatory organs of juvenile salmon in freshwater after 72 hours of urinary catheterization.** Letters indicate significant differences within each diet group. An asterisk (*) indicates a significant difference between low and medium salt diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

### 3.2.4 Hematology

*Blood pH*

The only significant difference observed was between the experimental and control low salt diet groups, with the experimental group High-A being 7.78±0.01 and significantly higher than the control group Control-A at 7.66±0.01 ($p<0.0168$, Fig. 20a).

*Hematocrit (%)*

The mean hematocrit was significantly different between control groups, with the low salt diet group Control-C being 27.5±1.2 % and significantly lower than both the medium salt diet group Control-B at 35.4±1.7 % ($p<0.0081$) and the high salt diet group Control-C at 33.6±1.1 % ($p<0.0228$) (Fig. 20b).

*Drabkin hemoglobin (g/dL)*

No significant differences in mean Drabkin Hb were observed between groups (Fig. 20c).

*Drabkin mean corpuscular hemoglobin concentration (MCHC)*

No significant difference in Drabkin MCHC was found between groups (Fig 20d).

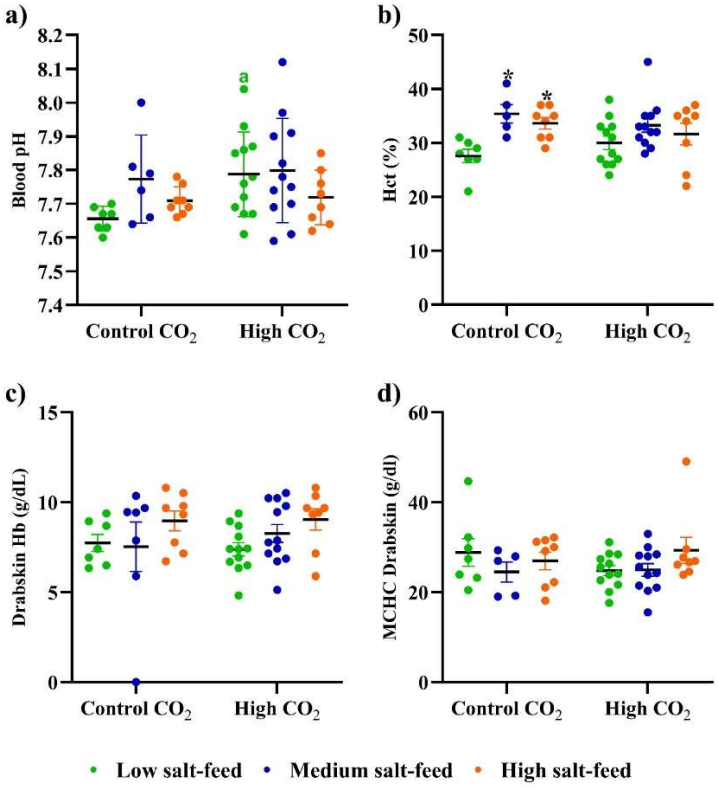


**Figure 20: Hematological parameters of juvenile salmon in freshwater after 72 hours of urinary catheterization.** Different letters indicate significant differences within each diet group. An asterisk (*) indicates a significant difference between low and medium salt diet groups. Individual data points are represented on the graphs, with mean and standard error of mean (SEM) represented by horizontal bars.

## 3.3 Nephrocalcinosis and water quality

### 3.3.1 Prevalence of nephrocalcinosis

The prevalence of nephrocalcinosis during this trial was higher for experimental groups, and prevalence increased with salt load in diets. For control groups, nephrocalcinosis was only detected for the control medium salt diet group Control-B with 8.33 % prevalence both pre- and 2W post-drop in the $CO_2$ drop trial and for the catheterization trial, with a total prevalence of 4.55% (Table 4). For experimental groups, the low salt diet group High-A had a prevalence of 8.33 % both at pre drop in the $CO_2$ drop trial and in the catheterization trial, with a total prevalence of 4.17 %, which was lower than Control-B due to a higher *n* (Table 4). The experimental medium salt diet group High-B had a prevalence of 8.33 % at pre-drop and to 41.67% at 5hr post-drop in the $CO_2$ drop trial, and a prevalence of 8.33 % in the catheterization trial, for a total prevalence of 14.58 % (Table 4). Finally, the experimental high salt diet group High-C had the highest prevalence with 58.33 % at pre-drop and 50.00 % at 5hr post-drop in the $CO_2$ drop trial, and 25.00 % in the catheterization trial, resulting in a total prevalence of 34.09 % (Table 4). Moderate cases of nephrocalcinosis with a macro score of 2 were only observed for High-B and High-C, with 4.17 % of the total 14.58 % for High-B, and 6.82 % of the total 34.09 % for High-C.

**Table 4: Prevalence (%) of nephrocalcinosis based on macro scoring for juvenile Atlantic salmon in freshwater (FW) with low (A), medium (B) and high (C) salt load in diets.** Overview of prevalence pre-drop at high $CO_2$ (30 mg/L) and after drop at control $CO_2$ (<8 mg/L) for high $CO_2$ groups (High), as well as 24 hours after drop for catheterized fish after three cycles of peak and drop. All prevalence were mild or moderate (macro score 1-2), with no cases of severe nephrocalcinosis.

| *Group / Time* | *n* | *Pre-drop* | *5hr post-drop* | *2W post-drop* | *n* | *Catheterization* | *Total (%)* |
|---|---|---|---|---|---|---|---|
| *Control-A* | 36 | 0 | 0 | 0 | 8 | 0 | 0 |
| *Control-B* | 36 | 8.33 | 0 | 8.33 | 8 | 0 | 4.55 |
| *Control-C* | 36 | 0 | 0 | 0 | 8 | 0 | 0 |
| *High-A* | 36 | 8.33 | 0 | 0 | 12 | 8.33 | 4.17 |
| *High-B* | 36 | 8.33 | 41.67 | 0 | 12 | 8.33 | 14.58 |
| *High-C* | 36 | 58.33 | 50.00 | 0 | 8 | 25.00 | 34.09 |

### 3.3.2 Water quality

The mean values show that temperature was approximately 12 °C, $O_2$ saturation was above 90%, while pH were approximately 7.2, and they were similar for all groups (Table 5). The mean concentration of $Ca^{2+}$ were generally higher for experimental groups at around 0.4 mg/L compared to control groups at slightly lower than 0.3 mg/L (Table 5). Meanwhile, mean

concentrations of $PO_4^{3-}$ and $Mg^{2+}$ increased with higher salt load in the diet, with concentrations being lowest for low salt diet groups, and highest for high salt diet groups (Table 5).

**Table 5:** Average water quality parameters in FW single RAS units (1 $m^3$) for experimental (High) and control (Control) groups with low (A), medium (B) and high (C) salt load (P, $Ca^{2+}$ and $Mg^{2+}$) in diets.

| *Group/parameter* | *Temperature (°C)* | *$O_2$ (%)* | *pH* | *[$PO_4^{3-}$] (mg/L)* | *[$Ca^{2+}$] (mg/L)* | *[$Mg^{2+}$] (mg/L)* |
|---|---|---|---|---|---|---|
| *Control-A* | 12.03±0.03 | 93.6±0.5 | 7.21±0.003 | 1.4±0.52 | 0.28±0.03 | 1.13±0.12 |
| *Control-B* | 12.19±0.04 | 91.5±0.5 | 7.21±0.001 | 4.2±1.18 | 0.29±0.02 | 2.10±0.31 |
| *Control-C* | 12.09±0.03 | 92.5±0.4 | 7.21±0.002 | 6.5±1.36 | 0.27±0.02 | 2.85±0.47 |
| *High-A* | 12.11±0.04 | 92.0±0.4 | 7.21±0.002 | 1.4±0.61 | 0.41±0.05 | 1.13±0.14 |
| *High-B* | 12.17±0.04 | 92.5±0.4 | 7.22±0.003 | 3.6±1.08 | 0.42±0.07 | 2.02±0.29 |
| *High-C* | 12.04±0.03 | 90.0±1.5 | 7.22±0.003 | 4.1±1.37 | 0.46±0.18 | 2.43±0.44 |

# 4. Discussion

## 4.1 Discussion of material and methods

### 4.1.1 Experimental design

Prior to trial entry, some fish were PIT-tagged, and all originated from the same tank. We would ideally have PIT-tagged all the fish entered in the trial to provide identical treatment for the whole population. However, only PIT-tagged fish were sampled so they could be identified and compared to baseline, and therefore the effect of unequal PIT-tagging should be minimal. The usage of single RAS units with two tanks per treatment group, instead of one RAS unit, increases the risk of slight differences in water quality between replicate units as each unit operates with its own separate biofilter. However, considering the measured water quality levels it seems that there were no big differences between replicate RAS units within and between groups. We did not see any difference in growth between replicate tanks, which can be observed in small scale tanks (Thorarensen, Kubiriza and Imsland, 2015). Nevertheless, growth performance was not the primary focus of this study.

Only water $CO_2$ levels were altered between experimental and control treatment groups, with $CO_2$ added to inlet water for experimental groups during ramp up to peak and peak $CO_2$ at approximately 30 mg/L $CO_2$. The pH was not affected as an automatic buffering system added NaOH buffer to the water and kept pH at 7.2, while water $O_2$ levels were kept above 80%

saturation for all tanks. Control $CO_2$ levels could only be kept below 8 mg/L due to biomass buildup, but should ideally stay lower, as even moderate amounts of $CO_2$ exposure have been shown to negatively affect the physiology and behavior of salmonids (Khan, Johansen and Skov, 2018). Additionally, when dropping $CO_2$ levels from peak, it took approximately 5 hours to reach control levels. Therefore, to better simulate transfers between tanks in commercial aquaculture facilities, where new tanks contain no accumulated $CO_2$ and the change in $CO_2$ level is immediate, lower control $CO_2$ levels and a quicker drop would be ideal. However, the drop delta was considered large and the drop duration short enough to trigger respiratory alkalosis, as fish spend up to 24 hours to downregulate bicarbonate concentrations before normal pH is restored (Hōbe, Wood and Wheatly, 1984; Wheatly, Hōbe and Wood, 1984; Perry, Malone and Ewing, 1987). The different durations of the 3 peak and drop cycles of $CO_2$ were based on the finding in two previous master theses' conducted at the Nofima research station in Sunndalsøra within the same project group. However, due to time restrictions and equipment failure we were unable to analyze the urine and blood parameters for the final sampling after 3 cycles in this thesis and the final sampling is thus not included here.

### 4.1.2 Sampling

Sampling was conducted in accordance with standardized protocols described in similar experiments (Curtis and Wood, 1991; McCormick, 1993; Sundell and Sundh, 2012; Takvam, Denker, *et al.*, 2021; Davison *et al.*, 2023; Takvam *et al.*, 2024). Handling of fish was based on the "*in-situ* anesthesia" method described by (Davison *et al.*, 2023) and modified to use buckets. During sampling, fish were anesthetized and always submerged in water, avoiding disturbance of acid-base blood parameters by capture stress and bradycardia associated with net-dipping, contrasting the "grab and stab" method, which will render acid-base parameters useless (Brauner *et al.*, 2019; Davison *et al.*, 2023). After blood, length, weight and urine sampling, the fish was always kept on ice until tissues were dissected and frozen on dry ice to prevent tissue degradation, and all sampling was conducted in a temperature-controlled room at 10-15 degrees Celsius.

Blood samples were extracted from the caudal vasculature within 5 minutes of adding anesthesia to bucket water as outlined by (Lawrence *et al.*, 2020). Arterial and venous vessels are closely situated in the caudal vasculature, and therefore blood obtained by caudal puncture is likely to result in a mixture of arterial and venous blood. This is not a problem for measurements of ions and circulatory proteins as they have a homogenous distribution in the circulatory system (Lawrence *et al.*, 2020). However, gas levels differ noticeably between

arterial and venous blood, and this method is less suited for measurements of partial pressure of respiratory gases like $O_2$ and $CO_2$ (Wood, McMahon and McDonald, 1979). Arterial cannulation would be the ideal method for accurate measurements of $PO_2$ and $PCO_2$ but was not feasible due to the sampling volume and experimental conditions, and therefore caudal puncture was used (Davison *et al.*, 2023).

Hb levels were measured using three methods: Drabkin and Austin (1935) haemoglobincyanide method, Hemocue 801 machine and Hemocue 201 machine. The haemoglobincyanide method is widely recognized as a highly accurate method for Hb estimation in human blood but requires working with hazardous reagents (cyanide compounds) and is time consuming (Blaxhall and Daisley, 1973; Srivastava *et al.*, 2014). The HemoCue machines are quick and easy to work with but are shown to both over- and underestimate Hb levels in human blood compared to the haemoglobincyanide method (Srivastava *et al.*, 2014). This was reflected during our experiment with both HemoCue machines yielding variable results compared to the haemoglobincyanide method. Hence, we based our results on the haemoglobincyanide method (Blaxhall and Daisley, 1973).

Prevalence of nephrocalcinosis was measured using macroscoring, as it is a quick and common method for determining the severity of the condition. Macroscoring can provide accurate results for more severe cases of nephrocalcinosis but tends to be inaccurate for mild cases. This is because mild cases with small precipitation can be difficult or even impossible to observe with the naked eye. Additionally, by macroscoring one is only observing the surface of the abdominal side of the kidney, while there could be precipitates further distal towards the spine. We did also sample kidney tissue for histopathology as this is regarded as the gold standard for scoring severity and can reliably detect mild cases of nephrocalcinosis. However, this method is resource intensive, time consuming and requires expertise to determine pathological histology. We could not get histopathological results in time for this thesis, due to the high sampling volume and time restrictions, and therefore the prevalence of nephrocalcinosis in this thesis is based on macroscoring alone.

### 4.1.3 Catheterization trial

In addition to the $CO_2$ drop trial, we also ran a catheterization trial to observe changes in urine during a drop in water $CO_2$ level. The fish was handled as described in section 4.1.2, and a catheter was surgically inserted and fastened as described by Curtis and Wood (1991), before the fish were put in catheterization chambers. This method is highly labor intensive compared

to urine spot sampling utilized in the $CO_2$ drop trial and requires expertise to not hurt the fish during operation. When dropping the $CO_2$ levels, the water supply from the main tank was shut off, and water within the bucket was circulated to a $CO_2$ stripper effectively aerating the water and removing $CO_2$ levels within half an hour. However, there was no make-up water or treatment of water quality except for the $CO_2$ stripper, so it's reasonable to assume some accumulation of metabolites and feces in the buckets for the last 24 hours of catheterization. Removing $CO_2$ also resulted in elevated pH in the buckets, as alkaline buffer was remaining in the water.

### 4.1.4 Statistical analysis

Due to the complexity of this experimental set-up (3 independent variables), we concluded that it would be reasonable to use both two-way and one-way ANOVAs. Two-way ANOVA was used for the independent variables $CO_2$ treatment (high and low) and salt loading in feed (low, medium and high), while one-way ANOVA was used for sampling times (1-3). Dependent variables in each analysis were measured parameters like animal performance, hematology, acid-base, NKA enzyme activity, ions and metabolites. ANOVA analysis was followed by Tukey's honestly significant difference (HSD) test, comparing mean values for each parameter between the independent variables. This test assumes that 1) the observations are independent, 2) normal distribution of dependent variables and 3) homogeneity of variance. Multiple comparisons are shown to increase the risk of Type 1 error (false positive), but Tukey's HSD minimizes this risk by adjusting p-values to correct for multiple comparisons (Nanda *et al.*, 2021). Using Tuckey's HSD test for a 95% confidence interval is therefore enabling better control over Type 1 errors, while improving detection of significant differences between and within groups.

## 4.2 Discussion of findings

The overall results of this study showed that fish exposed to high $CO_2$ had a higher risk of developing nephrocalcinosis, and that salt load in feed could have been a compounding effect. There have been several studies on the impact of sustained hypercapnia on development of nephrocalcinosis in salmonids (Fivelstad *et al.*, 1999, 2003; Hosfeld *et al.*, 2008), and the compensation for induced respiratory acidosis. However, few studies have focused on the effect of rapid changes in water quality on the acid-base status of the fish and its significance for developing kidney stones. As shown in the three articles by Wood and co-writers (1984), they conducted trials on respiratory acidosis induced by hyperoxia for rainbow trout and found that fish were able to compensate during hyperoxia by accumulating $HCO_3^-$ but struggled to

downregulate when rapidly returned to normoxia. Similar results were observed for this study demonstrating that there is a lag in downregulation of $HCO_3^-$ and that respiratory alkalosis induced by a return to normocapnia therefore causes a rise in urine pH that remains elevated for several hours. Additionally, prevalence of nephrocalcinosis increased with salt load in the diet for fish exposed to high $CO_2$, indicating a compounding effect on development of kidney stones as suggested by Takvam *et al.* (2023), as calcium, magnesium and phosphate are main constituents in formation of mineral precipitates in the kidney. The combination of elevated pH and ion availability in the urine therefore creates ideal conditions and increases risk for precipitation, as the rate of precipitation for kidney stones typically found in salmonids increases with increasing pH (Olszynski, Prywer and Torzewska, 2015).

### 4.2.1 Animal performance

#### *Growth*

Body mass and specific growth rate (SGR) were lower, but not significant, for all experimental groups compared to respective control groups in the $CO_2$ drop trial, which suggests that growth performance was negatively affected by exposure to high $CO_2$ levels (Fig. 5). This was further validated in the catheterization trial, after 2 more cycles of high $CO_2$ with longer peak periods (3 and 4 weeks), showing significantly lower body mass, SGR and condition factor (CF) for all experimental groups compared to control (Fig. 18). Several studies report reduced growth for salmonids exposed to hypercapnic rearing conditions (Fivelstad *et al.*, 1999, 2003, 2007; Hosfeld *et al.*, 2008; Khan, Johansen and Skov, 2018; Mota *et al.*, 2019), suggesting that appetite is suppressed and results in reduced growth. We could not confirm this, as fish were fed in excess throughout the duration of the trial, and the feed uptake was not measured. Another possibility could be that more energy is allocated to maintain ion- and acid-base regulation, which would otherwise be spent on growth. Additionally, the results show that groups with high salt diets showcased the greatest reduction in growth, scoring lowest for all growth parameters, which is consistent with the article of Basulto (1976) who found that inclusion of higher salt content in feed caused impairment of growth, and suggested it was caused by a dilution effect or by salt interfering with nutrient uptake.

#### *Nephrocalcinosis*

The overall prevalence of nephrocalcinosis in this study was higher for fish exposed to high $CO_2$, which is consistent with previous research (Fivelstad *et al.*, 1999, 2003; Hosfeld *et al.*, 2008). Additionally, higher salt load in the feed resulted in a clearly higher prevalence for fish exposed to high $CO_2$ as suggested by (Takvam *et al.*, 2023). Prevalence was highest at high

$CO_2$ and 5hr post-drop, with no prevalence for fish exposed to high $CO_2$ at 2W post-drop, indicating that fish struggled to acclimatize immediately after dropping $CO_2$ levels but recovered after two weeks. Interestingly, prevalence for the experimental group with medium salt diet increased from 8.33 % at high $CO_2$ to 41.67 % five hours after dropping $CO_2$ levels, suggesting that a rapid change in water quality caused physiological perturbations leading to mineral precipitation in the kidneys, which fits well with the suggested "drop hypothesis" of Takvam *et al.* (2023). In contrast, the "drop effect" on prevalence was not seen for the experimental groups with low and high salt diets, which decreased immediately after dropping $CO_2$ levels. However, the experimental high salt diet group still had a prevalence of 50 %, suggesting that the effect of high $CO_2$ is impacting the fish several hours after returning to normocapnia.

The majority of nephrocalcinosis observed in this study were mild cases with a macro score of 1, while only a few cases were moderate with a macro score of 2. Macro scoring can be inaccurate for mild cases of nephrocalcinosis as small precipitations can be hard to detect with the naked eye, and therefore it is possible that the real prevalence is higher and that this could be detected by utilizing histopathology. Despite this, clear differences between treatments were observed.

### *Stress response*

Stress is a factor that can affect important variables (blood gases, acid-base variables, hematology and stress hormones) when trying to obtain valid blood samples and determine the acid-base status of fish (Davison *et al.*, 2023). Barton and Iwama (1991) highlighted that stress response from handling causes increased cortisol, which then triggers an increase in glucose and muscular activity as a secondary response, which in turn makes glucose and lactic acid good stress indicators. Pickering, Pottinger and Christie (1982) found that the secondary responses did not necessarily occur simultaneously in brown trout (*Salmo trutta*), with glucose levels being delayed, peaking four hours after handling, while cortisol and lactic acid levels had returned to resting levels by that time. This suggests that lactic acid might be a better indicator of stress response from handling as samples are taken only minutes after anesthetization. Klykken *et al.* (2022) have suggested normal ranges for both glucose and lactic acid levels in plasma, with a range of 3.7-8.7 for glucose and 2.1-10.1 for lactate acid. The exact sampling method was not disclosed, but one can assume that sampling occurred by dip-netting fish in rearing tanks, as is the most common method. In this study, plasma glucose for all groups was similar and within the suggested normal ranges, indicating that fish exposed to high $CO_2$ had

acclimatized to the hypercapnic environment, and that a potential stress response due to dropping $CO_2$ levels had already passed before the 5hr post-drop sampling. Lactic acid levels between groups were also similar but were slightly below the suggested normal ranges, suggesting very low stress levels and that using the modified "*in-situ* anesthesia" method proposed by (Davison *et al.*, 2023) was efficient in minimizing handling stress.

### 4.2.2 Acid-base

This study clearly shows that rapid changes in $CO_2$ levels cause perturbations for acid-base regulation. The experimental groups had significantly higher $HCO_3^-$ concentrations in both plasma and urine during exposure to high $CO_2$, and levels decreased five hours after dropping $CO_2$ levels, but plasma concentrations remained elevated compared to control. This same effect was reported by Wood and co-writers in their three articles (1984), who investigated the effect of respiratory acidosis induced by hyperoxia and found that accumulation of $HCO_3^-$ was the main compensatory mechanism for regulating acid-base balance. They also found that during normoxic recovery, $PCO_2$ rapidly decreased while it took up to 24 hours before $HCO_3^-$ was regulated to normoxic levels, therefore inducing respiratory alkalosis and a rise in pH. This was further validated by the urinary catheterization trial where pH increased significantly and remained elevated for several hours (Fig. 19) after dropping $CO_2$ levels for experimental groups. However, this effect on pH was not found during the $CO_2$ drop trial, where blood and urine pH did not significantly change for fish exposed to high $CO_2$, despite $HCO_3^-$ levels remaining elevated. The drop in the urinary catheterization trial lasted approximately half an hour and $CO_2$ levels dropped below 1 mg/L, while the drop in the $CO_2$ drop trial was much slower, lasting 4-5 hours, and $CO_2$ were dropped to control levels (<8 mg/L). It is therefore possible that a quicker drop in $CO_2$ with a greater difference in $CO_2$ levels yielded a stronger response due to the lag in $HCO_3^-$ downregulation and excretion. A slow drop with gradually decreasing $CO_2$ levels might then provide enough time to dampen the response as fish are able to compensate within this timeframe, either by the excretion of $HCO_3^-$ or by regulation of ions like potassium, phosphate and calcium which are important for buffer capacity. Curiously, plasma $PCO_2$ for experimental groups in the $CO_2$ drop trial was only slightly higher than control groups when exposed to high $CO_2$, with a significant difference in $PCO_2$ levels only for high salt diet groups. This is likely due to the sampling technique, as blood sampling by caudal puncture is prone to collecting a mixture of arterial and venous blood, which differs regarding blood gas concentrations and can therefore be unreliable for measurements of blood gases like $O_2$ and $CO_2$. A more precise method for blood sampling of respiratory gases would be arterial

cannulation, but this introduces stress which affects other acid-base parameters, requires trained personnel and is highly labor intensive.

Despite the lack of a shift in blood and urine pH for fish exposed to elevated $CO_2$ in the $CO_2$ drop trial, the results show that hypercapnia caused elevated pH levels. Wood and co-writers found in their three articles (1984), that fish were able to completely compensate for the increase in $PCO_2$, however, the results of this study suggest a slight overcompensation. Another possibility was proposed by Eddy, Smart and Bath (1979) that exposed rainbow trout to high $CO_2$ and found that fish with nephrocalcinosis had unusually high values for urine pH, bicarbonate and urine/plasma bicarbonate ratio. They suggested that hypercapnia could interfere with the acidifying secretion of hydrogen ions and the reabsorption of filtered bicarbonate due to high plasma and cellular bicarbonate concentrations. This would therefore disrupt normal tubular function in the kidneys causing the production of alkaline pre-urine and urine, creating favorable conditions for precipitation of calcium phosphate deposits. The result of the present study is unclear as to whether it is hypercapnia or the rapid return to normocapnia that is triggering development of nephrocalcinosis, as prevalence was high for experimental groups both before and five hours after dropping $CO_2$ levels in the $CO_2$ drop trial. However, the $PCO_2$ levels in urine were much lower than plasma levels, which suggests that a disproportional amount of $HCO_3^-$ exists in urine compared to $PCO_2$ during hypercapnia, and consequently increasing urine pH. Unfortunately, urine $PCO_2$ and $HCO_3^-$ were not measured for the urinary catheterization trial, which would have been interesting to see considering the clear increase in urine pH observed immediately after dropping $CO_2$ levels. It is difficult to explain why the pH response was so different in the two trials of this study, however it is reasonable to believe that the differing drop methods would have an impact. For future studies on acid-base regulation, one should consider using the quicker drop method of the urinary catheterization trial, as it is the one most applicable to industry practice regarding handling procedures where the change in water quality is immediate when fish are transferred to new tanks.

### 4.2.3 Hematology

The results of this study showed only transient effects of changing $CO_2$ levels on hematocrit (Hct), hemoglobin (Hb) or mean corpuscular hemoglobin (MCHC) levels, indicating that elevated $CO_2$ levels had no long-term effect on $O_2$ uptake. In the $CO_2$ drop trial, Hct levels in fish exposed to high $CO_2$ dropped slightly 5 hours after drop, but was regulated back to control levels after 2 weeks. Fivelstad *et al.* (2003) observed similar results with Hct and Hb levels mostly unaffected, but with a decrease in MCHC after continuous exposure to high $CO_2$ (16

and 24 mg/L for 60 days) due to erythrocyte swelling. However, no such effect was observed in this study, as the combined $CO_2$ accumulation and peak periods for each cycle were considerably shorter (28, 35 and 42 days), suggesting repeated shorter periods of increased $CO_2$ have a lower risk of developing erythrocyte swelling as opposed to a longer period of continuously elevated CO2. Curiously, the low salt control groups had Hct levels significantly lower than the medium and high salt control groups after catheterization but appear to maintain oxygen carrying capacity as Hb levels were unaffected and MCHC was slightly higher. The reason for this is uncertain as diet did not appear to have an effect in any other groups, however low availability of ions might disturb the production of erythrocytes or affect the release or storage of erythrocytes in the spleen.

### 4.2.4 Ion regulation in plasma and urine

Acid-base balance and ionoregulation are tightly linked as the major organs responsible for ion regulation are also important for uptake and excretion of acid-base equivalents, as described in previous studies (Hōbe, Wood and Wheatly, 1984; Wheatly, Hōbe and Wood, 1984; Wood, Wheatly and Hōbe, 1984; Klykken *et al.*, 2022; Zimmer and Perry, 2022; Takvam *et al.*, 2023). A disturbance in acid-base status might then lead to altered ion concentrations as fish compensate to maintain internal pH. Ion regulation and regulation of acid-base equivalents is largely handled by the gills while the rest is mostly handled by the kidney (Takvam *et al.*, 2023). Despite this, Klykken *et al.* (2022) found that plasma chloride and sodium levels were reduced for Atlantic salmon with severe nephrocalcinosis, suggesting that the kidney damage can cause ionoregulatory perturbations and cause altered plasma ion concentrations as it is important for reabsorption of ions in FW. Ion concentrations in this study were only measured for the $CO_2$ drop trial and not in the urinary catheterization trial, as equipment was unavailable and time was too limited regarding the completion of this thesis. Therefore, this section will focus on the immediate effects of dropping $CO_2$ levels from high (30 mg/L) to control (<8 mg/L) after the first cycle (14-day accumulation + 14-day peak), and not the long-term effects after 3 cycles.

#### *Monovalent ions*

Normal levels of monovalent ions in FW teleost plasma is reported by Takvam, *et al.* (2021) to be approximately 154 mM for $Na^+$, 134 mM for $Cl^-$, and 2.4 mM for $K^+$, while normal urine concentrations are approximately 28 mM for $Na^+$, 6.4 mM for $Cl^-$ and 2.1 for $K^+$. Sodium and chloride are commonly described to be affected by respiratory acid-base disturbances, as $Na^+$ is exchanged for the acid equivalents $H^+$ and $NH_4^+$, while $Cl^-$ is exchanged for the basic equivalents $OH^-$ and $HCO_3^-$ (Takvam *et al.*, 2023). Several studies found that respiratory

acidosis caused a decrease in pH which was compensated largely by branchial $Cl^-/HCO_3^-$ exchange, causing a decrease in plasma $Cl^-$ levels and equivalent increase in $HCO_3^-$ levels, while $Na^+$ uptake is increased while acidic equivalents are excreted. Additionally, Wood, Wheatly and Hōbe, (1984) found that respiratory alkalosis caused a net excretion of $Na^+$ and a net uptake of $Cl^-$, hypothesizing that the mechanism is reversible.

While trials on short term $CO_2$ exposure (up to 7 days) for salmonids have demonstrated an increase in plasma $Na^+$ levels in response to respiratory acidosis (Eddy, Smart and Bath, 1979; Wood, Wheatly and Hōbe, 1984), trials on long term $CO_2$ exposure (42-384 days) found no change in, or even a slight reduction (Hosfeld *et al.*, 2008; Good *et al.*, 2018). This suggests that plasma $Na^+$ levels might be affected in the short term but appears to be restored as fish acclimatize to hypercapnia. Indeed, fish exposed to high $CO_2$ in this study had similar plasma $Na^+$ levels compared to control, suggesting that a gradual increase in environmental $CO_2$ over 14 days followed by a 14-day peak period allowed the fish to acclimatize. However, plasma $Na^+$ levels were expected to decrease following a return to normocapnia and induced respiratory alkalosis, but no such effect was observed. Additionally, there was even a slight significant increase in plasma $Na^+$ levels two weeks after dropping $CO_2$, but this increase occurred in both experimental and control groups, albeit the experimental groups with medium and high salt diet being higher. Looking at the urine $Na^+$ levels, there were no significant changes before or after dropping $CO_2$ levels for experimental groups. However, the urine $Na^+$ levels were slightly higher compared to control groups, indicating increased $Na^+$ excretion by the kidney which is supported by slightly higher UP ratios. Reabsorption of $HCO_3^-$ is described to be dependent on secretion of $H^+$ via a $Na^+/H^+$ exchanger and $Na^+/HCO_3^-$ co-transporter in the kidney tubules during respiratory acidosis (Takvam *et al.*, 2023), but as $HCO_3^-$ must be excreted and $H^+$ conserved during respiratory alkalosis, reabsorption of $Na^+$ might be reduced causing an increase in excretion. Another possibility could be related to the onset of nephrocalcinosis, as even mild cases of nephrocalcinosis could cause structural damage to kidney tubule tissues and therefore alter reabsorption ability. This could also explain the increase in plasma $Na^+$ concentration two weeks after dropping $CO_2$ levels, as there were no cases of nephrocalcinosis for the experimental groups at this time, suggesting that kidney function was restored.

The effects of acid-base perturbations are clearer for plasma $Cl^-$ levels, as both short- and long-term $CO_2$ exposure trials report a decrease in $Cl^-$, linked to the accumulation of $HCO_3^-$, when fish are subjected to respiratory acidosis (Hōbe, Wood and Wheatly, 1984; Wheatly, Hōbe and Wood, 1984; Wood, Wheatly and Hōbe, 1984; Fivelstad *et al.*, 1999, 2003; Hosfeld *et al.*,

2008). The findings of this study seem to coincide with previous literature as plasma $Cl^-$ levels were slightly lower for fish exposed to high $CO_2$, while a significant increase was observed two weeks after dropping $CO_2$ levels, indicating that $Cl^-$ and $HCO_3^-$ are closely linked through the $Cl^-/HCO_3^-$ - anion exchanger. Wood, Wheatly and Hōbe (1984) also observed increased $Cl^-$ excretion at the start of normoxic recovery despite respiratory alkalosis, and hypothesized it being due to diffusive "leaks" as FW fish are hyperosmotic to its environment. This could explain why no influx of chloride ions was observed five hours after dropping $CO_2$ levels. The urine $Cl^-$ levels were significantly higher for fish exposed to high $CO_2$ during hypercapnia, indicating higher excretion. There is no net uptake of ions and solutes in the kidney, unlike for the gills, as it can only reabsorb filtrate from the plasma. During respiratory acidosis plasma $Cl^-$ is lost due to $HCO_3^-$ uptake at the gills, and it is therefore vital that $Cl^-$ is reabsorbed in the kidney during respiratory acidosis. Chloride reabsorption is not dependent on $HCO_3^-$ exchange in the kidneys, but is suggested to occur firstly through kidney specific apical $Na^+/K^+/Cl^-$ co-transporter (NKCC2) found in the distal tubule and $Na^+/Cl^-$ co-transporter (NCC) found in the collecting duct, and then through basolateral specific $Cl^-$ channel (ClC-K), which all utilizes the transepithelial membrane potential created by the NKA pump (Takvam, Wood, *et al.*, 2021). The high $Cl^-$ levels observed in the urine are therefore contradictory to what is expected for a FW acclimated fish, as it need to create highly diluted urine to maintain ionoregulatory balance. This again points to possible structural damage caused by nephrocalcinosis as $Cl^-$ reabsorption is reduced. Interestingly, the high salt diet group was the least affected of the experimental groups despite having the highest prevalence of nephrocalcinosis, while the opposite occurred for the low salt diet group. However, urine $Cl^-$ levels returned to control after dropping $CO_2$ for experimental groups, suggesting that there were no long-term effects.

Potassium is a vital cation in vertebrates as basolateral NKA pumps maintain high intracellular $K^+$ levels and creates the membrane potential utilized by secondary transporters but knowledge on regulatory mechanisms of plasma and urine $K^+$ levels in fish remain limited. Wood, Wheatly and Hōbe (1984) reported an initial increase in brachial efflux of $K^+$ levels when respiratory acidosis was induced that eventually decreased, but surprisingly a further increase occurred when fish entered normoxic recovery. In contrast, Hosfeld *et al.* (2008) found no change in plasma $K^+$ levels during hypercapnia, while Dimberg and Höglund (1987) observed an increase in plasma $K^+$ levels. In this study, plasma $K^+$ levels were similar between experimental and control groups, but concentrations appeared to increase with lower salt load in the diets. However, $K^+$ levels were slightly below the reported normal levels of 2.4 mM by Takvam,

Wood, *et al.* (2021), but within the normal range suggested for Atlantic salmon of 1.5-4.9 mM by Klykken *et al.* (2022). Exposure to high $CO_2$ did not seem to affect the plasma $K^+$ levels except for a significant difference for low salt diet groups at 5hr post-drop. Potassium concentrations in urine were almost identical to $Cl^-$ levels, being considerably higher for experimental low and medium salt diet groups during hypercapnia. Uptake of $K^+$ is suggested to occur through the NKCC2 and NCC transporters and the ClC-K channel during NaCl reabsorption, while excretion would occur through the basolateral NKA pump and a apical $K^+$ channel (ROMK2) (Takvam, Wood, *et al.*, 2021). Wheatly, Hōbe and Wood (1984) describes a charge balance in the urine, and one could therefore speculate that the high $K^+$ levels is a response to high $Cl^-$ levels, as their concentrations within groups are very similar.

### *Divalent ions*

Elevated $Ca^{2+}$ and $Mg^{2+}$ levels are suggested to increase the risk of developing nephrocalcinosis as these ions are main constituents of kidney stones typically found in salmonids (Fivelstad *et al.*, 2003; Klykken *et al.*, 2022, 2023; Takvam *et al.*, 2023). However, regarding acid-base disturbances, divalent ions like $Ca^{2+}$ and $Mg^{2+}$ have been considered to a lesser extent, and findings are varied. In previous research, increased $Ca^{2+}$ levels have been observed in kidney during hypercapnia (Fivelstad *et al.*, 2003) and in plasma during hyperoxia, which interestingly increased further during normoxic recovery (Wheatly, Hōbe and Wood, 1984). In contrast, Good *et al.* (2018) found no difference in whole blood $Ca^{2+}$, and Skov (2019) concluded that high $CO_2$ alone do not cause an increase in whole body or whole blood content of $Ca^{2+}$. However, Wood, Wheatly and Hōbe (1984) found increased branchial $Ca^{2+}$ uptake during hyperoxia and linked its importance in achieving ionic net charge balance. Normal levels of $Ca^{2+}$ in teleost fish is reported to be 1.5 mM in plasma and 1.1 mM in urine, while normal $Mg^{2+}$ levels are 1.3 mM in plasma and 0.42 mM in urine (Takvam, Wood, *et al.*, 2021). However, the ranges of 2.1-3.4 mM for $Ca^{2+}$ and 0.7-1.5 mM for $Mg^{2+}$ in Atlantic salmon plasma suggested by Klykken *et al.* (2022) is more appropriate as both experimental and control groups had higher $Ca^{2+}$ and slightly lower $Mg^{2+}$ than the proposed normal levels. Despite Smart *et al.* (1979) observing an increase in kidney $Mg^{2+}$, $Ca^{2+}$ and phosphorus (P) levels for rainbow trout exposed to high $CO_2$, findings regarding $Mg^{2+}$ levels in FW salmonids are sparse. It would therefore seem that the exact effect of acid-base disturbances on $Ca^{2+}$ and $Mg^{2+}$ is poorly understood.

In this study, increased in plasma $Ca^{2+}$ levels seemed to coincide with the prevalence of nephrocalcinosis, while plasma $Mg^{2+}$ appeared unaffected. The experimental groups had significantly higher plasma $Ca^{2+}$ levels, while also having the highest prevalence during

hypercapnia. Klykken *et al.* (2022) observed similar results with elevated plasma $Ca^{2+}$ levels for fish with nephrocalcinosis but could not determine whether the high plasma $Ca^{2+}$ detected were the cause of or caused by the disorder. Additionally, two weeks after dropping $CO_2$ levels both plasma $Ca^{2+}$ and prevalence of nephrocalcinosis decreased to control levels, indicating that mild cases are reversible when returned to normocapnia. Klykken *et al.* (2022) also observed higher $Mg^{2+}$ levels for fish with moderate and severe cases of nephrocalcinosis, suggesting that it was caused by kidney damage. Nephrocalcinosis observed in this study were mostly mild cases (macro score of 1), with a few moderate cases (macro score of 2), which might explain why plasma $Mg^{2+}$ observed for fish exposed to high $CO_2$ were similar to those of the control.

Urine $Ca^{2+}$ and $Mg^{2+}$ levels had some variation but were relatively close to normal reported values both during hypercapnia and five hours after dropping $CO_2$ levels. However, concentrations increased significantly for both experimental and control medium and high salt diet groups two weeks after dropping $CO_2$. Considering that this effect was observed in both experimental and control, it is unlikely that high $CO_2$ levels or onset of nephrocalcinosis was the cause. Regardless of the cause, the effect seemed to increase with salt load in the diet as the high salt diet groups were most affected.

### *Inorganic phosphate*

There is limited literature regarding normal values for inorganic phosphate in salmonids, but Klykken *et al.* (2022) have suggested an interval of 2.4 to 8.1 mM in plasma for Atlantic salmon. The plasma phosphate levels measured in this study were at the lower end of this interval or slightly below, with clear differences between salt diets. Minarova *et al.* (2023) observed reduced plasma phosphate for fish exposed to hypercapnia and suggested that respiratory acidosis induced by hypercapnia causes $PO_4^{3-}$ to be excreted in the kidney. No such response was observed in this study, but plasma phosphate levels slightly decreased after dropping $CO_2$ levels and inducing respiratory alkalosis for experimental groups. However, plasma concentrations of inorganic phosphate seemed highly dependent on the phosphate levels in their respective diets. Phosphate levels in the urine were lower for fish who had been exposed to high $CO_2$ and increased slightly after two weeks but remained below control levels, and this could be due to high reabsorption as both UP ratios and FE rates were low for experimental groups. A possible cause for this would be mineral precipitation in the kidney, as plasma levels did not increase and phosphate is one of the main components of kidney stones in salmonids.

### 4.2.5 The impact of salt on osmoregulation

Special feeds, also referred to as salt feeds, typically contain a mixture of $Ca^{2+}$, $Mg^{2+}$, P, NaCl, and amino acids, and are utilized by the aquaculture industry for the purpose of achieving better smolt quality and stimulation of SW tolerance. Previous research on salt feeds have largely been focused on dietary NaCl, showing that salmonids fed a diet containing 5-10% dietary NaCl had increased amounts of chloride cells, increased gill NKA enzyme activity and SW tolerance (Basulto, 1976; Zaugg *et al.*, 1983; N. A. Salman and Eddy, 1988; N. A Salman and Eddy, 1988; Duston, 1993; Perry *et al.*, 2006), and that changes appear similar to responses described in gills and hypoosmotic regulation by exposure to increased salinity in the water (McCormick, 2001). A general trend from the literature is that increased inclusion of dietary salts causes reduced growth and has been attributed to the dilution effect on other nutrients in the feed. However, Duston (1993) found a 60-70% reduction in growth for Atlantic salmon by the inclusion of 10% NaCl in the diet, which could not be explained by a dilution effect. Additionally, higher salt content in feed can also cause increased blood pressure (Olson and Hoagland, 2008) and increased load on the kidneys (Takvam, Wood, *et al.*, 2021), and therefore increase the risk of physiological disturbances and conditions like nephrocalcinosis. Even though salt feeds have been shown to have a positive effect in combination with light regimes, the research is sparse on whether elevated dietary salts like $Ca^{2+}$, $Mg^{2+}$, and minerals like P, may be a risk factor for impaired osmoregulatory function and development of nephrocalcinosis.

Ionic calcium is described to be strictly regulated in fish, and $Ca^{2+}$ homeostasis in FW is maintained by active branchial and intestinal $Ca^{2+}$ absorption through a basolateral ATP-dependent $Ca^{2+}$ pump or by a $Na^{+}/Ca^{2+}$ exchanger powered by the membrane potential created by NKA (Lall, 2021). There is limited knowledge on the impact of elevated levels of dietary $Ca^{2+}$ in the feed. In FW, $Mg^{2+}$ is absorbed by active uptake both from the environment and from dietary sources, but unlike $Ca^{2+}$ uptake, sufficient uptake of $Mg^{2+}$ is dependent on dietary absorption (Lall, 2021). Additionally, while the absorption mechanisms of $Mg^{2+}$ have received little attention, Oikari and Rankin (1985) found an increase in renal tubular secretion of $Mg^{2+}$ for FW rainbow trout with magnesium salts infused into the body cavity, and suggested that excess $Mg^{2+}$ were excreted renally by FW fish.

In this study, both $Ca^{2+}$ and $Mg^{2+}$ levels in plasma were similar between diet groups, indicating efficient regulation of plasma concentrations despite differing salt loads. The UP ratio and FE rate of $Ca^{2+}$ were low before and five hours after dropping $CO_2$ levels, indicating high renal

reabsorption. Meanwhile, FE rate for $Mg^{2+}$ was also low, indicating high reabsorption, while UP ratio was high, indicating low reabsorption and complete excretion for some groups. Creatinine levels in the urine were considerably higher than in the plasma before dropping $CO_2$ levels, which would explain the low FE rate, as it considers the clearance rate for creatinine to determine the reabsorption/excretion of a given ion. Creatinine is eliminated as filtrate through the glomerulus and by secretion in proximal tubules (Kashani, Rosner and Ostermann, 2020) and elevated creatinine levels could be an indication of increased GFR. This is however highly speculative, as GFR and UFR were not measured during the $CO_2$ drop trial, and UFR was found to be similar between all groups during the catheterization trial, suggesting that there was no change. Another possibility is that fish were stressed as creatinine secretion is affected by both stress and fish size, as it is the end product of creatine and creatinine phosphate metabolism in muscle cells (Kashani, Rosner and Ostermann, 2020). Two weeks after dropping $CO_2$ levels UP and FE rates for both $Ca^{2+}$ and $Mg^{2+}$ increased significantly for medium and high salt diet groups, indicating reduced reabsorption and increased excretion by the kidney. A possible explanation is increased uptake of divalent ions in the intestine as intestinal NKA enzyme activity was elevated, and therefore excess ions were renally excreted to maintain plasma levels. However, the cause of this effect remains unclear and considering that there was no difference found between diet groups prior to this sampling, there must be some other factor triggering this response, though it appears that higher salt load in the diet compounds this effect. It seems unlikely that the onset of nephrocalcinosis is causing this disturbance, as control groups were also affected despite low observed prevalence of the disorder. Further research into the effect of $Ca^{2+}$ and $Mg^{2+}$ load on osmoregulatory capacity and its implications for onset of nephrocalcinosis is needed.

Phosphate has received some attention where Ketola and Richmond (1994) found that discharge of phosphorus in effluent water increased with increasing levels of dietary P for rainbow trout. This was also observed with Drábiková *et al.* (2023) who found that the fibroblast growth hormone *fgf23*, which suppresses renal phosphate reabsorption, was upregulated in response to increased phosphate levels in feed, while the opposite occurred for low phosphate levels within dietary requirements. This suggests that phosphate levels are maintained by renal regulation, as an increase in phosphate levels causes corresponding decrease in reabsorption. However, in the present study, both UP ratios and FE rates varied greatly between diet groups, with low salt diet groups having the lowest reabsorption rates at the pre-drop sampling, while at the 2W post-drop sampling, the high salt diet groups had very low reabsorption rates or complete secretion. The

results are conflicting and the sudden increase in UP ratios and FE rates for medium and high salt diet groups remains uncertain.

### 4.2.6 NKA enzyme activity

The NKA enzyme is vital in maintaining the transmembrane potential in cells, which is utilized for secondary transport of ions and solutes against their gradients (Skou and Esmann, 1992). In this study, NKA activity remained stable for gills and kidney during the $CO_2$ drop trial, despite large changes in water $CO_2$ level, which is surprising considering that these are the main sites for regulation of mono- and divalent ions and acid-base equivalents (Evans, Piermarini and Choe, 2005). The NKA activity in proximal and distal intestine increased significantly after dropping $CO_2$ levels for experimental groups. Interestingly, control groups had increased enzyme activity as well, despite not being subjected to any environmental change. The exact cause of this is unclear, as one would expect control groups to remain stable, but this suggests that there is some other factor than $CO_2$ levels influencing both groups. Additionally, higher salt load groups had lager increases in intestinal NKA enzyme activity, indicating that there might be some feed related effects. The effects seen in intestine tissues could also be a sampling error, as tissue samples are prone to degradation due to digestive proteins and should be analyzed within 48 hours of sampling.

# 5. Conclusion

This study shows that rapid changes in rearing water from high to low $CO_2$ levels induces respiratory alkalosis and increases urine pH, which combined with increased availability of ions from feed, creates ideal conditions for mineral precipitation and onset of nephrocalcinosis. The main aim of this study was to increase our knowledge of physiological changes caused by acid-base perturbations due to changes in environmental $CO_2$ levels and different salt load in feeds.

**Objective 1: Determine NKA enzyme activity in the three main osmoregulatory organs before and after dropping $CO_2$ levels in rearing tanks.**

Overall NKA enzyme activity in gills and kidney remined stable, contrasting the assumption of increased enzyme activity related to perturbation of acid-base and ion regulation. Intestinal NKA enzyme activity increased after dropping $CO_2$ levels but could not be attributed to acid-base disturbance as control groups were affected. The increase in enzyme activity increased with salt load in diets, but the cause was not determined, and further research is needed.

**Objective 2: Compare acid-base status before and after dropping $CO_2$ levels in rearing tanks.**

As predicted, plasma [$HCO_3^-$] was accumulated in response to respiratory acidosis induced by elevated $CO_2$ levels. After dropping $CO_2$ levels respiratory alkalosis was induced due to slow downregulation of $HCO_3^-$, which corresponds with previous research on salmonids. However, a slower drop reaching normocapnic environments after 5 hours yielded no significant changes in plasma and urine pH, despite elevated [$HCO_3^-$], while a rapid drop lasting 30 minutes caused a significant disturbance in acid-base regulation and increased urine pH which remained elevated for 24 hours. Two weeks after returning to normocapnic environments normal acid-base status was restored. Future research should emphasize quick transitions from hypercapnic to normocapnic environments to better simulate industry handling procedures.

**Objective 3: Determine changes in renal handling of ions when acid-base regulation is disturbed, and the effect of different salt load in feeds.**

Elevated $CO_2$ caused higher excretion of monovalent ions ($Na^+$, $Cl^-$, $K^+$), while increased reabsorption occurred after dropping $CO_2$ levels, which deviates from findings in current literature. Excretion of $Mg^{2+}$ and was generally high, likely due to excess uptake from feed. Low excretion of $Ca^{2+}$ and $PO_4^{3-}$, and high $Ca^{2+}$ plasma levels, for fish exposed to elevated $CO_2$ coincided with prevalence of nephrocalcinosis, which have been reported in previous studies, while excretion increased after dropping $CO_2$ levels. Excretion of $Cl^-$ and $K^+$ increased with decreased salt load in diets, while excretion of divalent ions increased with increasing salt load.

**Objective 4: Determine the effects of rearing environments with rapidly changing $CO_2$ levels and different salt loads in feed on animal performance and prevalence of nephrocalcinosis.**

Elevated $CO_2$ and higher salt load in diets caused reduced growth and increased the risk of developing nephrocalcinosis, which is consistent with previous research on salmonids. However, prevalence increased for one group and decreased for other groups after dropping $CO_2$ levels, and development of nephrocalcinosis could not be attributed to the drop alone, which deviates from the initial predictions.

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# Appendix A:

**Table A.1: Significant changes in NKA activity in juvenile Atlantic salmon pre-, 5hr- and 2W post-drop, with experimental groups (High) going from high $CO_2$ (30 mg/L) to control $CO_2$ (<8 mg/L) levels.** Different letters indicate significant differences within groups ($p<0.05$).

| $Na^+/K^+$ ATPase | | | | | | |
|---|---|---|---|---|---|---|
| Group / Time | **Control-A** | **Control-B** | **Control-C** | **High-A** | **High-B** | **High-C** |
| Proximal intestine NKA | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | B | B | A | A | B | A |
| 2w post-drop | C | C | B | B | C | B |
| Distal intestine NKA | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | B | A | B | A | A | A |
| 2w post-drop | AB | B | B | B | B | B |
| Gill NKA | | | | | | |
| Pre-drop | A | A | A | A | A | AB |
| 5hr post-drop | B | A | B | A | A | A |
| 2w post-drop | AB | A | A | A | B | B |
| Kidney NKA | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | B | A | A | B | A |
| 2w post-drop | A | AB | A | B | A | A |

**Table A.2: Significant changes in hematological parameters in juvenile Atlantic salmon pre-, 5hr- and 2W post-drop, with experimental groups (High) going from high $CO_2$ (30 mg/L) to control $CO_2$ (<8 mg/L) levels.** Different letters indicate significant differences within groups ($p<0.05$).

| Hematological parameters | | | | | | |
|---|---|---|---|---|---|---|
| Group / Time | **Control-A** | **Control-B** | **Control-C** | **High-A** | **High-B** | **High-C** |
| Hct % | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | AB | A | B |
| 2w post-drop | A | A | A | B | A | AB |
| Drabkin Hb (g/dL) | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | A | A | A |
| 2w post-drop | A | A | A | A | A | A |
| Drabkin MCHC | | | | | | |
| Pre-drop | A | AB | AB | A | A | A |
| 5hr post-drop | A | A | A | A | A | A |
| 2w post-drop | A | B | B | A | A | A |

**Table A.3: Significant changes in growth parameters in juvenile Atlantic salmon pre-, 5hr- and 2W post-drop, with experimental groups (High) going from high $CO_2$ (30 mg/L) to control $CO_2$ (<8 mg/L) levels.** Different letters indicate significant differences within groups ($p<0.05$).

| Animal performance | | | | | | |
|---|---|---|---|---|---|---|
| Group / Time | **Control-A** | **Control-B** | **Control-C** | **High-A** | **High-B** | **High-C** |
| Body mass (g) | | | | | | |
| Pre-drop | A | A | A | A | A | AB |
| 5hr post-drop | A | A | A | AB | A | A |
| 2w post-drop | A | A | B | B | A | B |
| Specific growth rate (SGR) | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | A | A | A |
| 2w post-drop | A | A | A | A | A | A |
| Condition factor (CF) | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | AB | A | A | AB | AB |
| 2w post-drop | A | B | B | A | B | B |

**Table A.4: Significant changes in plasma acid-base parameters in juvenile Atlantic salmon pre-, 5hr- and 2W post-drop, with experimental groups (High) going from high $CO_2$ (30 mg/L) to control $CO_2$ (<8 mg/L) levels.** Different letters indicate significant differences within groups ($p<0.05$).

| Plasma Acid-Base | | | | | | |
|---|---|---|---|---|---|---|
| Group / Time | **Control-A** | **Control-B** | **Control-C** | **High-A** | **High-B** | **High-C** |
| Blood pH | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | A | A | A |
| 2w post-drop | B | B | B | A | A | A |
| Plasma $[HCO_3^-]$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | AB | B | B | B | B | B |
| 2w post-drop | B | AB | A | C | C | C |
| Plasma $P_aCO_2$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | B | AB | A | B | B |
| 2w post-drop | B | C | B | B | C | B |
| Plasma $TCO_2$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | B | B | B | B | B |
| 2w post-drop | B | AB | A | C | C | C |

**Table A.5: Significant changes in urine acid-base parameter in juvenile Atlantic salmon pre-, 5hr- and 2W post-drop, with experimental groups (High) going from high $CO_2$ (30 mg/L) to control $CO_2$ (<8 mg/L) levels.** Different letters indicate significant differences within groups ($p<0.05$).

| Urine Acid-Base | | | | | | |
|---|---|---|---|---|---|---|
| Group / Time | **Control-A** | **Control-B** | **Control-C** | **High-A** | **High-B** | **High-C** |
| Urine pH | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | A | A | A |
| 2w post-drop | A | A | A | A | B | A |
| Urine $[HCO_3^-]$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | B |
| 2w post-drop | B | A | B | AB | B | C |
| Urine $PCO_2$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | AB | B |
| 2w post-drop | B | A | A | B | B | C |
| Urine $TCO_2$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | B |
| 2w post-drop | B | A | B | AB | B | C |

**Table A.6: Significant changes in plasma ions and metabolites in juvenile Atlantic salmon pre-, 5hr- and 2W post-drop, with experimental groups (High) going from high $CO_2$ (30 mg/L) to control $CO_2$ (<8 mg/L) levels.** Different letters indicate significant differences within groups ($p<0.05$).

| Plasma ions and metabolites | | | | | | |
|---|---|---|---|---|---|---|
| Group / Time | **Control-A** | **Control-B** | **Control-C** | **High-A** | **High-B** | **High-C** |
| Plasma $[Na^+]$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | AB | AB | A | A | A | A |
| 2w post-drop | B | B | B | A | B | B |
| Plasma $[Cl^-]$ | | | | | | |
| Pre-drop | AB | A | A | AB | A | A |
| 5hr post-drop | A | A | A | A | A | A |
| 2w post-drop | B | A | A | B | B | B |
| Plasma $[K^+]$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | B | A | A | B | AB | A |
| 2w post-drop | A | A | A | B | B | A |
| Plasma $[Mg^{2+}]$ | | | | | | |
| Pre-drop | A | A | A | A | A | AB |
| 5hr post-drop | A | A | B | A | A | A |
| 2w post-drop | A | A | B | A | A | B |
| Plasma $[Ca^{2+}]$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |

| | | | | | | |
|---|---|---|---|---|---|---|
| 5hr post-drop | A | A | A | A | AB | B |
| 2w post-drop | A | A | A | B | B | C |
| Plasma [$PO_4^{3-}$] | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | B | A |
| 2w post-drop | A | A | A | A | A | A |
| Plasma total protein | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | B | A | B | B | A | A |
| 2w post-drop | C | A | A | A | A | B |
| Plasma creatinine | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | B | A | A | A | A |
| 2w post-drop | A | A | B | A | B | B |
| Plasma glucose | | | | | | |
| Pre-drop | A | A | A | AB | AB | A |
| 5hr post-drop | AB | AB | A | A | A | A |
| 2w post-drop | B | B | A | B | B | B |
| Plasma albumin | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | A |
| 2w post-drop | A | A | A | C | B | B |
| Plasma lactic acid | | | | | | |
| Pre-drop | A | A | A | AB | A | A |
| 5hr post-drop | A | A | A | A | A | A |
| 2w post-drop | A | A | A | B | A | A |

**Table A.7: Significant changes in urine plasma and metabolites in juvenile Atlantic salmon pre-, 5hr- and 2W post-drop, with experimental groups (High) going from high $CO_2$ (30 mg/L) to control $CO_2$ (<8 mg/L) levels.** Different letters indicate significant differences within groups ($p<0.05$).

| **Urine ions and metabolites** | | | | | | |
|---|---|---|---|---|---|---|
| Group / Time | **Control-A** | **Control-B** | **Control-C** | **High-A** | **High-B** | **High-C** |
| Urine [$Na^+$] | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | A |
| 2w post-drop | A | A | A | AB | B | A |
| Urine [$Cl^-$] | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | A |
| 2w post-drop | A | A | A | A | A | A |
| Urine [$K^+$] | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | A |
| 2w post-drop | A | A | A | B | A | A |
| Urine [$Mg^{2+}$] | | | | | | |
| Pre-drop | A | A | A | A | A | AB |
| 5hr post-drop | AB | A | A | B | A | A |

| | | | | | | |
|---|---|---|---|---|---|---|
| 2w post-drop | B | B | B | A | B | B |
| Urine [$Ca^{2+}$] | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | AB | A | A | B | A | A |
| 2w post-drop | B | B | B | C | B | B |
| Urine [$PO_4^{3-}$] | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | A |
| 2w post-drop | A | A | B | A | B | B |
| Urine total protein | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | B | A | A | A |
| 2w post-drop | A | A | B | A | B | B |
| Urine creatinine | | | | | | |
| Pre-drop | AB | A | A | A | A | A |
| 5hr post-drop | A | A | B | B | A | A |
| 2w post-drop | B | A | A | A | A | A |
| Urine albumin | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | B | A | A | A |
| 2w post-drop | A | A | B | A | A | B |

**Table A.8: Significant changes in urine-plasma ratios for ions, metabolites and acid-base parameters in juvenile Atlantic salmon pre-, 5hr- and 2W post-drop, with experimental groups (High) going from high $CO_2$ (30 mg/L) to control $CO_2$ (<8 mg/L) levels.** Different letters indicate significant differences within groups ($p<0.05$).

| **Urine-Plasma (UP) ratios** | | | | | | |
|---|---|---|---|---|---|---|
| Group / Time | **Control-A** | **Control-B** | **Control-C** | **High-A** | **High-B** | **High-C** |
| [$Na^+$] | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | A |
| 2w post-drop | A | A | A | AB | B | A |
| [$Cl^-$] | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | A |
| 2w post-drop | A | A | A | AB | B | A |
| [$K^+$] | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | B | A | A | B | AB | A |
| 2w post-drop | A | A | A | B | B | A |
| [$Mg^{2+}$] | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | AB | B | A | B | A | A |
| 2w post-drop | B | B | A | A | B | B |
| [$Ca^{2+}$] | | | | | | |
| Pre-drop | A | A | A | A | A | A |

| | | | | | | |
|---|---|---|---|---|---|---|
| 5hr post-drop | AB | A | A | B | A | A |
| 2w post-drop | B | B | B | C | B | B |
| $[PO_4^{3-}]$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | A |
| 2w post-drop | A | A | B | A | B | B |
| Albumin | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | A | A | AB |
| 2w post-drop | A | A | A | A | A | B |
| CREA4 | | | | | | |
| Pre-drop | A | A | A | AB | A | A |
| 5hr post-drop | A | A | B | A | A | A |
| 2w post-drop | A | A | B | B | A | B |
| $TCO_2$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | A |
| 2w post-drop | B | A | B | A | B | B |
| $PCO_2$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | A |
| 2w post-drop | B | A | A | A | A | B |
| $[HCO_3^-]$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | B | A | A | B | A | A |
| 2w post-drop | A | A | B | A | B | B |

**Table A.9: Significant changes in urine-plasma fractional excretion rate for ions, albumin and acid-base parameters in juvenile Atlantic salmon pre-, 5hr- and 2W post-drop, with experimental groups (High) going from high $CO_2$ (30 mg/L) to control $CO_2$ (<8 mg/L) levels.** Different letters indicate significant differences within groups ($p<0.05$).

| **Urine-Plasma fractional excretion (FE) rate** | | | | | | |
|---|---|---|---|---|---|---|
| Group / Time | **Control-A** | **Control-B** | **Control-C** | **High-A** | **High-B** | **High-C** |
| $[Na^+]$ | | | | | | |
| Pre-drop | AB | A | A | AB | A | A |
| 5hr post-drop | A | A | B | A | A | B |
| 2w post-drop | B | A | B | B | A | A |
| $[Cl^-]$ | | | | | | |
| Pre-drop | AB | A | A | A | A | AB |
| 5hr post-drop | A | A | B | A | A | A |
| 2w post-drop | B | A | B | A | A | B |
| $[K^+]$ | | | | | | |
| Pre-drop | AB | A | A | A | A | A |
| 5hr post-drop | A | A | A | AB | A | B |
| 2w post-drop | B | A | A | B | A | A |
| $[Mg^{2+}]$ | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | AB | B | A | A | B |
| 2w post-drop | A | B | B | A | B | C |
| $[Ca^{2+}]$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | B | A | A | A |
| 2w post-drop | A | B | C | A | B | B |
| $[PO_4^{3-}]$ | | | | | | |
| Pre-drop | A | AB | A | A | A | A |
| 5hr post-drop | A | A | A | A | A | B |
| 2w post-drop | A | B | B | A | A | C |
| Albumin | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | A | A | B |
| 2w post-drop | A | A | A | A | A | B |
| $TCO_2$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | B | A | A | B |
| 2w post-drop | A | A | A | A | B | B |
| $PCO_2$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | B | A | A | AB |
| 2w post-drop | B | A | B | A | A | B |
| $[HCO_3^-]$ | | | | | | |
| Pre-drop | A | A | A | A | A | A |
| 5hr post-drop | A | A | A | B | A | A |
| 2w post-drop | A | A | B | A | B | B |