

OXALATE HANDLING AND TRANSPORTER EXPRESSION IN *POECILIA LATIPINNA* IN
RESPONSE TO ENVIRONMENTAL SALINITY AND ANTIBIOTIC EXPOSURE

FELICIA RAJAN

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGY
YORK UNIVERSITY
TORONTO, ONTARIO

AUGUST 2022

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ABSTRACT

Oxalate is obtained either through the diet or as a by-product of metabolism and the primary route of excretion is via the kidney. However, salinity may pose a challenge for urinary oxalate excretion in seawater teleosts and thus, the intestine may play an enhanced role in this environment. Little is known about oxalate transport in fish, however mammalian models suggest the involvement of solute carrier family transporters (SLC), specifically SLC26A3 and SLC26A6. Further, mammalian research highlights an important role of gut microbiota. To study the effects of salinity and bacteria on intestinal oxalate excretion, sailfin mollies (*Poecilia latipinna*) were acclimated to freshwater or seawater (for either 14 or 28 days) and seawater for 14 days with broad-range antibiotic treatment. Initial acclimation to seawater increased the plasma oxalate concentration but homeostasis was recovered by 28 days. Kidney oxalate concentrations were unaffected by salinity, however the concentration in the anterior intestine increased in seawater while it decreased in the posterior intestine. Further, SLC26A3 expression in both the anterior and posterior intestine was downregulated in response to seawater acclimation, while SLC26A6 expression was upregulated after seawater acclimation for 14 days. Antibiotics reduced the expression of both SLC26A6 and SLC26A3 in both intestinal sections and altered the concentrations of oxalate along the intestine. These findings suggest that the anterior intestine may play an enhanced role in oxalate regulation in seawater. Further, the kidneys may play a significant role in handling oxalate with high levels of SLC26A6 expression when compensating for the loss of oxalate-degrading bacteria in the gut.

ACKNOWLEDGEMENTS

Firstly, I would like to wholeheartedly thank my supervisor, Dr. Carol Bucking for all her help, support, and guidance during my studies. Dr. Bucking's encouragement during the past two years have been invaluable to me. Thank you for helping me trouble-shoot my experiments and teaching me to think critically about all aspects of the scientific method. I am grateful to Dr. Bucking for all the opportunities she provided me to improve my skills as a researcher.

I would also like to thank my advisor, Dr. Raymond Kwong for always providing me with useful feedback that improved not only my experiments but also my writing and presentation skills. The members of the Kwong Lab, especially Preeti and Theanuga were the best neighbors anyone could ask for. Thank you for being so kind and welcoming to me when I joined the Bucking Lab.

My lab-mate, Jacob is also owed many thanks for helping me acclimate to the lab setting when I first started my studies. Over the past two years we've made some unforgettable memories both in the lab and at Starbucks. Thank you for being a wonderful lab-mate and an even more amazing friend.

My mom is owed the biggest "thank you" of all because it was her constant love and support that enabled me to pursue my dreams of going to graduate school. Thank you for having faith in me. I genuinely appreciate everything you have done for me and how you have always encouraged my passion for school. Thank you for not only being an absolutely incredible mom but also for being my best friend.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
cDNA	Complementary deoxyribonucleic acid
gDNA	Genomic deoxyribonucleic acid
DNA	Deoxyribonucleic acid
FW	Freshwater
MS-222	Tricane methanesulfonate
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RPL7	Ribosomal protein L7
RPL17	Ribosomal protein L17
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SEM	Standard error of mean
SLC26A3	Solute carrier family 26, member 3
SLC26A6	Solute carrier family 26, member 6
SW-14	Seawater acclimation – 14 days
SW-28	Seawater acclimation – 28 days
TAE	Tris-acetate-EDTA (TAE) buffer
UV	Ultraviolet
16S	16S ribosomal RNA gene
18S	18S ribosomal RNA gene

CHAPTER 1: GENERAL BACKGROUND

1.1 Osmo- and iono-regulation in teleosts

Osmoregulation is the process of regulating water potential to maintain fluid balance within an organism (Evans *et al.*, 2005). Relatedly, ionoregulation is an organism's homeostatic maintenance of the concentrations of various ions (e.g. Na^+ , Cl^- , Ca^{2+} , K^+ , Mg^{2+}) in bodily fluids (Molnar & Gair, 2019). In particular, fish generally maintain an internal osmotic pressure of ~ 290 mOsm L^{-1} and specific internal ion concentrations regardless of the surrounding salinity (Table 1.1). There are different capabilities within species of fish to withstand environmental ionic strengths and several terms that describe the ability of fish to survive in different salinities. Stenohaline fish are unable to withstand wide variation in the salinity of their surrounding waters and generally only inhabit a single salinity over their life history (Gilbert, 2017). Examples include: goldfish (*Carassius auratus*) which is a stenohaline freshwater fish, and haddock (*Melanogrammus aeglefinus*) which is a stenohaline seawater fish. In contrast, euryhaline fish can live in waters of a wide range of salinity (Gilbert, 2017), often quickly acclimating to environmental salinity challenges. Examples include: striped bass (*Morone saxatilis*), Asian sea bass (*Lates calcarifer*), and sailfin molly (*Poecilia latipinna*), all of which are capable of inhabiting a variety of environmental salinities. Combined, this results in environmentally- and species-specific osmotic and ionic challenges when the surrounding water salinity is altered.

Specifically, in freshwater, the plasma ion content of teleosts is hypertonic compared to their environment (Table 1.1) and thus, they passively lose ions by diffusion down the concentration gradient (Figure 1.1; Greenwell *et al.*, 2003). Further, as teleosts' body fluids are hyperosmotic compared to their freshwater environments (Table 1.1), there is a constant influx of water (Figure 1.1; Greenwell *et al.*, 2003). Thus, fish in freshwater face the challenge of potential

overhydration (hypervolemia) and ion depletion (Greenwell *et al.*, 2003). To combat these challenges, fish have adapted several mechanisms and characteristics to excrete water and gain ions. First, their skin is scaly and covered with a protective mucous, reducing its permeability, and thus the teleost skin is a very minor site of water and ion flux (Greenwell *et al.*, 2003). In contrast, there is a large influx of water across their gill epithelium (Kultz, 2015), and loss of ions (Beyenbach, 2004). In fact, unidirectional water fluxes across the gill epithelium can be as high as 50% of the total body water per hour (Greenwell *et al.*, 2003). Further, ion loss across the gill can account for up to 63% of ion losses (Walker, 1971). To maintain homeostasis, teleosts inhabiting freshwater drink very little of their surrounding water and excrete large quantities of dilute urine (Kultz, 2015). In particular, the teleost kidney is tasked with eliminating large quantities of water while limiting ion loss through reabsorption of ions lost in the glomerular filtrate (Figure 1.1; Kultz, 2015). High glomerular filtration rates are coupled with an almost complete renal reabsorption of Na^+ and Cl^- to allow for efficient water secretion and salt retention (Greenwell *et al.*, 2003). Finally, freshwater gills are equipped with transporters that maintain active ion absorption against the ion gradient from the water (Figure 1.1; Hwang *et al.*, 2011).

Contrarily, in seawater, teleosts inhabit an environment that is hyperosmotic compared to their body fluids (Table 1.1) and thus, face dehydration (hypovolemia; Figure 1.1). Further, the internal ion content is much lower than the surrounding seawater (Table 1.1) and fish face constant ion loading (e.g. hypernatremia and hyperchloremia; Figure 1.1; Greenwell *et al.*, 2003). Thus for seawater teleosts, the main goal of all osmotic and ionic processes is to gain water and fight ion loading (Kultz, 2015). Specifically, teleosts in seawater gain salts like NaCl by passive diffusion down the ion concentration gradient and lose water via osmosis (Table 1.1; Kultz, 2015). Similar to freshwater teleosts, their impermeable skin has minimal involvement in water flux (Greenwell

et al., 2003), while the gills appear to be the key organs involved in detrimental water and ion fluxes (Figure 1.1; Kultz, 2015). To maintain homeostasis, seawater fish drink large amounts of seawater and subsequently perform a series of ion exchanges and ion secretions to keep their body fluids more dilute than their environment (Figure 1.1; Greenwell *et al.*, 2003). Teleosts living in moderately saline waters will drink 10-20% of their body weight each day but can drink up to 35-40% of their body weight per day in hypersaline waters (Greenwell *et al.*, 2003). Following imbibition, the esophagus and intestine absorb and/or precipitate approximately 60-80% of the seawater components drunk by seawater teleosts (Greenwell *et al.*, 2003; Grosell *et al.*, 2018) to lower the osmotic pressure in the intestinal lumen, and in turn drive osmotic water absorption in the gut (Figure 1.1). Specifically, K^+ , Na^+ , and Cl^- ions are first absorbed by the seawater teleost's esophagus, thereby lowering the osmotic pressure of the imbibed seawater (Grosell *et al.*, 2018). These excess ions are in turn excreted via specialized chloride cells in the gills, operculum, and skin on the head of most seawater teleost species (Figure 1.1; Greenwell *et al.*, 2003). Notably, these secretory pathways also combat passive ion gain by diffusion from the environment. Further, Ca^{2+} (and to a lesser extent Mg^{2+}) forms inorganic mineral precipitates such as calcium carbonate ($CaCO_3$) and calcium oxalate (CaC_2O_4) in the intestine (Kurita *et al.*, 2008; Grosell *et al.*, 2009; reviewed by Whittamore, 2020). This precipitation further lowers the luminal osmotic pressure and drives additional water absorption (Figure 1.1; Grosell *et al.*, 2018). Altogether, the reduction in osmotic pressure in the gut (through monovalent ion absorption and Ca^{2+} precipitation) allows for water uptake through osmosis (Grosell *et al.*, 2018) while active secretion of ions at the gills fights ion gain (Figure 1.1). Finally, the absorbed water is highly conserved within the body fluids of seawater teleosts and is reabsorbed by the urinary bladder (Greenwell *et al.*, 2003). Further, seawater teleosts produce very small volumes of concentrated urine and their daily urine flow rate

is only 1-2% of their body weight (Figure 1.1; Greenwell *et al.*, 2003). Interestingly, the kidney appears to be the primary site of divalent ion secretion (Beyenbach, 1995; reviewed by Watanabe & Takei, 2011) while the gills appear to be primarily active in monovalent ion unloading in a seawater environment (Marshall & Grosell, 2006; Edwards & Marshall, 2013; reviewed by Ferreira-Martins *et al.*, 2021).

HCO_3^- secretion in the intestine plays a crucial role in osmoregulation by lowering the pH in the intestine and aiding in the precipitation of a large amount of the imbibed calcium as calcium carbonate (Grosell & Wilson, 2003). Indeed, the high levels of calcium (e.g. 20 mM) in the intestine of seawater teleosts along with the high pH results in approximately 75% of the calcium found in the intestine being precipitated as calcium carbonate (Grosell & Wilson, 2003). This precipitation drives water absorption as discussed above but also reduces diffusive absorption of calcium thus protecting the seawater teleost kidney from the need for excessive calcium excretion and possibly increased risk of kidney stone (calcium oxalate) formation (Grosell & Wilson, 2003). The proximal intestine of seawater teleosts has been observed to have the highest rates of HCO_3^- secretion via the chloride/bicarbonate ($\text{Cl}^-/\text{HCO}_3^-$) anion exchangers from the SLC26 gene family, particularly SLC26A3 and SLC26A6 (Grosell *et al.*, 2009; Gregorio *et al.*, 2013; Alves *et al.*, 2019; reviewed by Whittamore, 2020). In addition to bicarbonate playing an instrumental role in osmo- and iono-regulation, oxalate may also play a role by forming additional Ca^{2+} precipitates in the intestine. However, the impact of the environment on oxalate production, transport, and tissue handling has so far been understudied in fish.

1.2 Role of oxalate in teleosts

Oxalate can be consumed exogenously via the diet, in particular from aquatic plants (e.g. *Cabomba*, *Egeria*, and *Limnophila*; Dyer, 2020). It is also a by-product of endogenous protein

breakdown in the liver (Asplin, 2002; reviewed by Whittamore, 2020) and both exogenous and endogenous sources of oxalate may equally contribute to urinary oxalate excretion (Holmes *et al.*, 2001). In the kidney, oxalate anions readily bind with calcium cations to form calcium oxalate. During hyperoxaluria, when the serum calcium oxalate concentration is higher than the saturation concentration, oxalate will begin to solidify out of the solution and can accumulate to form kidney stones (Asplin, 2002; Li, 2017). In fact, accumulation of oxalate can result in oxalosis, kidney stones, tubular necrosis, kidney destruction, and blindness in fish (Blazer & Wolke, 1983). Environmental factors like salinity can affect calcium oxalate crystal formation (Wilson & Grosell, 2003; reviewed by Whittamore, 2020). Specifically, in freshwater, oxalate is usually filtered out through the kidney and excreted in the large amount of dilute urine (Figure 1.1). Since teleosts living in seawater excrete extremely small volumes of urine, a build-up of oxalate can occur and this toxic compound poses a critical risk to these fish's renal health (Figure 1.1; Kultz, 2015).

Not much is known about oxalate transport in fish, but research in mice (*Mus musculus*) has identified several key transporters. Specifically, using knockout mice, SLC26A3 was shown to be involved in transcellular oxalate absorption and SLC26A6 was found to be vital for intestinal oxalate secretion (Freel *et al.*, 2006, 2013). It is thus possible that intestinal transport of oxalate in seawater teleosts may involve these same proteins and molecular pathways (Freel *et al.*, 2006, 2013, reviewed by Whittamore, 2020). Both transporters are involved in intestinal water absorption in seawater teleosts (Ruhr *et al.*, 2016; Ando *et al.*, 2014; reviewed by Alves *et al.*, 2019) due to their roles in HCO_3^- secretion as mentioned above. Remarkably, microorganisms in the mammalian gut have been shown to play a key role in the pathogenesis and prevention of kidney stones (Mehta *et al.*, 2016). In mammals, *Oxalobacter formigenes* degrades large amounts of oxalate in the gut and decreases the amount of urinary oxalate excretion (Hatch *et al.*, 2006). In

fish, Whittamore (2020) proposed that the teleost gut is a major excretory pathway for oxalate based on very large secretion rates observed in the mammalian (particularly mouse and human) intestine (Whittamore & Hatch, 2017). Past studies have not examined the role of the fish gut microbiome in oxalate degradation.

Our current lack of knowledge surrounding oxalate in fish, including handling, transport, and bacterial degradation, may be critically impacting our understanding of fish physiology. The importance of this lack of understanding is illustrated in aquaculture. According to a 2020 report by the Food and Agriculture Organization of the United Nations, aquaculture accounted for 46% of the global fish production in 2018. Aquaculture is also expected to increase in the future due to a growing population and increased demand for seafood (Hardy, 2010; Hixson, 2014). Obtaining fishmeal when farming carnivorous and/or omnivorous species of fish not only taxes the environment with the need for wild fish but is also expensive (Naylor *et al.*, 2000). For both increased sustainability and profitability, the aquaculture field is trying to reduce the quantity of fishmeal and fish oil in their aquafeeds by supplementing it with plants (Caruso, 2015). Therefore, protein sources from plants are being utilized in either partial or total replacement of fishmeal in aquaculture practices (Hardy, 2010). The oxalate concentration of soybean seeds from 11 different cultivars had relatively high levels of total oxalate, ranging from 0.67 to 3.5 g/100g of dry weight (Massey *et al.*, 2001). Therefore, an increase in using plants such as the soybean, which is already widely used as a fishmeal alternative in aquaculture practices (Brown *et al.*, 2008), may result in the farmed fish experiencing negative health impacts due to the increased levels of oxalate being consumed through their plant-enriched diets.

1.3 Thesis Rationale:

Overall, salinity is an important abiotic factor for fish that determines their osmo- and iono-regulation strategies (Zhang *et al.*, 2017). However, the impact of salinity on oxalate and its potential role in maintaining water homeostasis in fish has received little scientific attention. In particular, the potential role of bacteria in intestinal oxalate homeostasis remains undiscovered. Sailfin mollies have been chosen as the subjects of this study as these euryhaline teleosts can tolerate salinities from 0-90 ppt (Gonzalez *et al.*, 2005). This makes them excellent models to explore the impact of salinity on physiological processes. Further, these animals are herbivores (Courtenay & Meffe, 1989), consuming primarily plant material which is comparatively rich in oxalate. This may be an important factor due to dietary dependent oxalate concentrations and will also serve as a novel comparison as the majority of past studies have been conducted on carnivorous fish.

1.3.1 Study objectives

The main objective of this study was to observe how an herbivorous euryhaline teleost, the sailfin molly (Courtenay & Meffe, 1989), handles oxalate, a potentially urotoxic compound when faced with the osmoregulatory challenges posed by salinity. The role of the kidney and intestine were compared due to their known role in mammalian oxalate regulation as well as the well-established roles in piscine salinity acclimation. Additionally, the role of the gut microbiome in oxalate degradation/transport was investigated. Specifically, the oxalate concentrations of the anterior intestine, posterior intestine, kidney, and plasma were measured across short-term and long-term salinity exposure conditions as well as following an antibiotic treatment. To better understand the effect of salinity and antibiotic treatment on oxalate transport within the intestines and kidneys, SLC26A3 and SLC26A6 mRNA expression was also measured.

This was the first study to directly measure oxalate concentrations in the plasma, kidneys, and intestines of an euryhaline teleost and thus, it increased our understanding of how these animals cope with this urotoxic compound. This study showed that both salinity and antibiotic treatment impacted the organism's overall ability to regulate oxalate. This study also increased our knowledge of how salinity and antibiotic treatment affected SLC26A3 and SLC26A6 expression in the kidneys and intestines of the sailfin molly.

Table 1.1: Examples of osmotic pressures and ion contents of both freshwater and seawater as well as the teleosts who inhabit these environments.

Habitat	Fish's Internal Composition	References
Freshwater (<0.5 ppt) Osmotic Pressure: <1 mOsm/kg H ₂ O Ion Content: Na ⁺ : 0.75 ± 0.15 mmol L ⁻¹ Cl ⁻ : 0.91 ± 0.12 mmol L ⁻¹ Ca ²⁺ : 1.16 ± 0.04 mmol L ⁻¹ K ⁺ : 0.08 ± 0.01 mmol L ⁻¹ Mg ²⁺ : 1.27 ± 0.12 mmol L ⁻¹	Freshwater Teleost Plasma Osmotic Pressure: 280 mOsm/kg H ₂ O Plasma Ion Content: Na ⁺ : 217.22 ± 2.70 mmol L ⁻¹ Cl ⁻ : 126.43 ± 3.31 mmol L ⁻¹ Ca ²⁺ : 1.43 ± 0.21 mmol L ⁻¹ K ⁺ : 14.39 ± 0.73 mmol L ⁻¹ Mg ²⁺ : 0.70 ± 0.06 mmol L ⁻¹	Osmotic Pressures: Beyenbach, 2004 Ion Contents: Becker <i>et al.</i> , 2011
Seawater (>30 ppt) Osmotic Pressure: ~1000 mOsm/kg H ₂ O Ion Content: Na ⁺ : 439.58 ± 5.12 mmol L ⁻¹ Cl ⁻ : 534.54 ± 6.25 mmol L ⁻¹ Ca ²⁺ : 16.10 ± 1.06 mmol L ⁻¹ K ⁺ : 13.90 ± 0.29 mmol L ⁻¹ Mg ²⁺ : 34.54 ± 0.35 mmol L ⁻¹	Seawater Teleost Plasma Osmotic Pressure: 350 mOsm/kg H ₂ O Plasma Ion Content: Na ⁺ : 235.47 ± 0.61 mmol L ⁻¹ Cl ⁻ : 185.30 ± 3.33 mmol L ⁻¹ Ca ²⁺ : 2.85 ± 0.11 mmol L ⁻¹ K ⁺ : 6.75 ± 0.40 mmol L ⁻¹ Mg ²⁺ : 1.56 ± 0.23 mmol L ⁻¹	Osmotic Pressures: Beyenbach, 2004 Ion Contents: Becker <i>et al.</i> , 2011

* Plasma ion contents above are for *Micropogonias furnieri* (Whitemouth drummer), an euryhaline teleost

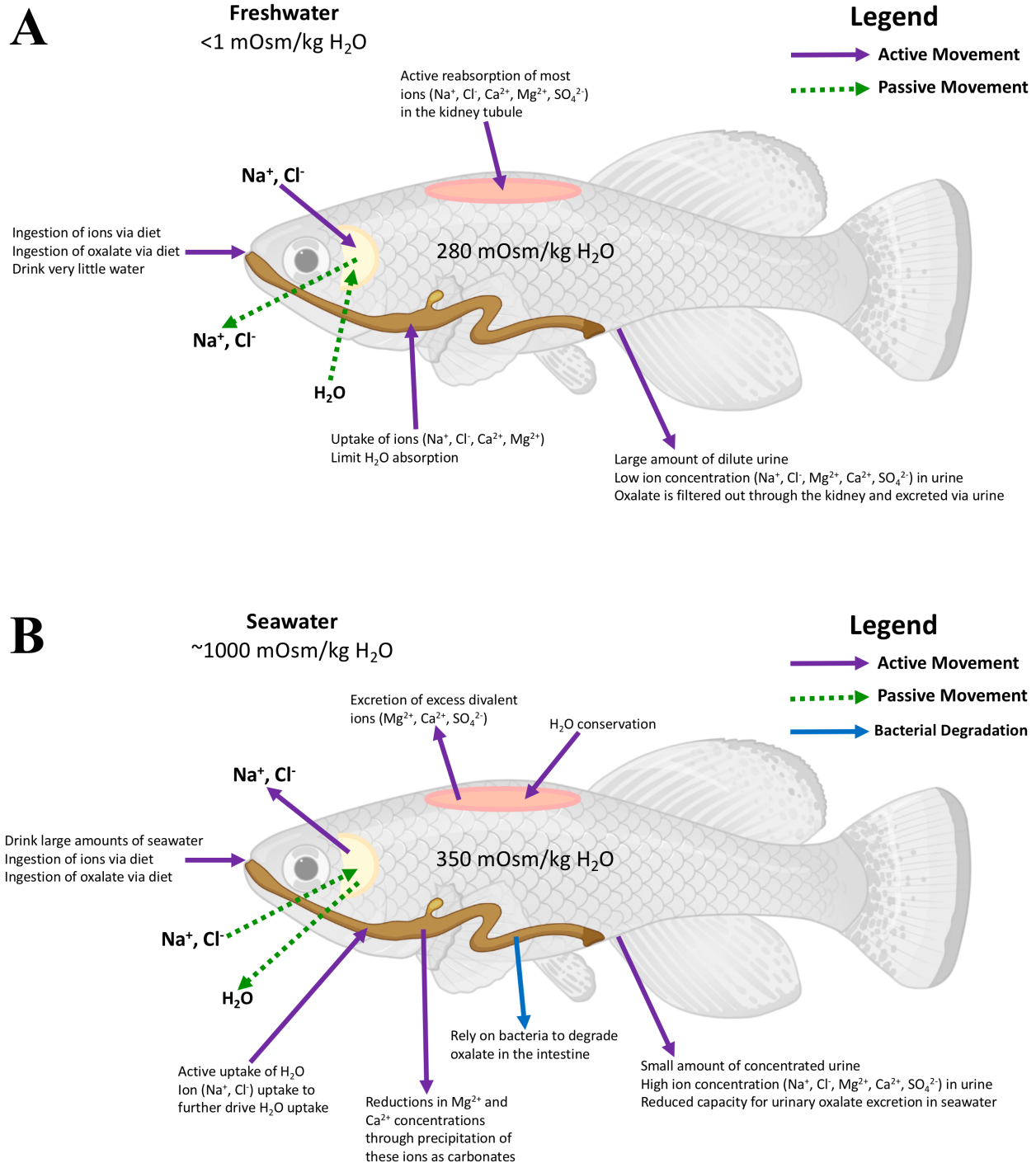


Figure 1.1 (A and B): A diagram of the iono-regulation and osmoregulation strategies employed by teleosts in freshwater (A) and seawater (B). These diagrams mainly focus upon ion and water transport in the gills (beige), kidneys (pink), and intestines (brown). These schematics also illustrate the way in which oxalate might be handled within the teleost's body as they become acclimated to these two different environmental conditions. See text for more details. These illustrations were created using biorender.com.

CHAPTER 2: THE EFFECT OF SALINITY ON OXALATE EXCRETION AND THE ROLE OF THE TELEOST INTESTINE AS A POTENTIAL EXCRETORY PATHWAY FOR OXALATE

2.1 INTRODUCTION

2.1.1 Oxalate

In vertebrates, oxalate can be consumed as part of the diet (i.e. exogenous source) and is also a non-functional by-product of metabolism (i.e. endogenous source; Knight *et al.*, 2011). In particular, there are many exogenous sources of dietary oxalate which include vegetables for mammals (e.g. spinach, rhubarb, and soya beans have high oxalate contents; Huang *et al.*, 2020) and aquatic plants for fish (e.g. *Cabomba*, *Egeria*, and *Limnophila*; Dyer, 2020). Further, endogenous sources of oxalate are primarily synthesized by the liver (Huang *et al.*, 2020) as a byproduct of amino acid metabolism including the breakdown of tyrosine, tryptophan, phenylalanine, and hydroxyproline (Fargue *et al.*, 2016). Additionally, the primary immediate precursor of oxalate is glyoxylate, an important intermediate in many reactions, which is metabolized by hepatic glycolate oxidase or lactate dehydrogenase into oxalate (Huang *et al.*, 2020). In mammals, excessive levels of glyoxylate in the liver will increase metabolism of glyoxylate to oxalate, which in turn will lead to an excessive urinary excretion of oxalate (i.e. hyperoxaluria; Huang *et al.*, 2020). Excessive dietary intake of oxalate in mammals can also lead to hyperoxaluria (Li, 2017). Regardless of the source of oxalate, urinary excretion is the primary mode of elimination in mammals (Holmes *et al.*, 2001).

Once filtered into the kidney, oxalate anions may readily precipitate out of solution potentially leading to physiological consequences (Asplin, 2002; reviewed by Whittamore, 2020).

Oxalate has been observed to form covalent bonds with divalent cations such as Ca^{2+} and Mg^{2+} and form compounds/crystals, which can detrimentally accumulate to form kidney stones (Asplin, 2002; Li, 2017). In fact, approximately 75% of mammalian kidney stones are composed primarily of calcium oxalate and thus, urinary oxalate concentration is deemed to be a crucial risk factor (Mehta *et al.*, 2016). While research into oxalate excretion, calcium oxalate crystal formation, and the effects of kidney stones on the organism's overall health has primarily focused on mammalian models a few studies have been conducted on fish (Whittamore & Hatch, 2017). Calcium oxalate crystal formation has a possible correlation to renal disease and blindness in fish (Blazer & Wolke, 1983). In a two-year study of adult clownfish (*Amphiprion ocellaris*), the only consistent finding that explained their blindness was the presence of calcium oxalate crystals in their kidney tubules. Specifically, these calcium oxalate crystals resulted in tubular necrosis and eventually caused kidney destruction (Blazer & Wolke, 1983). Secondly, we also know that the process of calcium oxalate crystal formation in fish is influenced by environmental factors such as salinity (Wilson & Grosell, 2003; reviewed by Whittamore, 2020). Normally in FW, oxalate is usually filtered out through the kidney and excreted in the copious amounts of urine (Figure 2.1). However, teleosts living in seawater excrete extremely small volumes of urine (Figure 2.1; Kultz, 2015). Thus, the build-up of this toxic compound poses a critical risk to these fish's renal health (Blazer & Wolke, 1983) and they may use the intestine as an alternative pathway (Whittamore, 2020).

2.1.2 Intestinal oxalate

Both mammals and fish appear to also rely on the intestine for oxalate excretion (Figure 2.1). In particular, the formation of kidney stones was associated with poor kidney function and a four-fold increase in fecal oxalate excretion in rats (*Rattus norvegicus*; Costello *et al.*, 1992). Further, seawater teleosts also have the ability to secrete oxalate in the intestine and may do so

preferentially as rates exceed 15-19 times greater than those observed in the mammalian small intestine (Hatch *et al.*, 1994; Whittamore & Hatch, 2017). How oxalate is transported and metabolized in the intestine may be critical knowledge for fish physiologists, however almost all published literature uses mammalian models.

2.1.2.1 Transport: Anion exchangers SLC26A3 and SLC26A6

There have been numerous mammalian studies showing the gut playing a role in oxalate metabolism by using oxalate transporters to absorb and excrete oxalate (Robijn *et al.*, 2011; Knight *et al.*, 2013; Miller *et al.*, 2019; Mehta *et al.*, 2016). Further, transporters of the SLC26 gene family have been identified as intestinal oxalate transporters. While the SLC26 family is composed of 10 different subtypes of anion exchangers involved in the transport of Cl^- , I^- , SO_4^{2-} , OH^- , HCO_3^- , formate, and oxalate (Mount & Romero, 2003), both SLC26A3 (Freel *et al.*, 2013) and SLC26A6 (Freel *et al.*, 2006) have been specifically observed to significantly affect oxalate transport within the mammalian intestine (reviewed by Whittamore & Hatch, 2017). Firstly, SLC26A6 is considered to be a major oxalate-excreting transporter that is predominantly expressed in the small intestine of mice (Liu *et al.*, 2021) but is also present in the apical membrane of the kidney's proximal tubule (Knauf *et al.*, 2001; reviewed by Wang *et al.*, 2005; Figure 2.2 A and B). SLC26A6 functions to exchange many anions, including: $\text{Cl}^-/\text{oxalate}$, $\text{oxalate}/\text{SO}_4^{2-}$, $\text{Cl}^-/\text{formate}$, $\text{oxalate}/\text{formate}$, $\text{oxalate}/\text{oxalate}$, Cl^-/OH^- , and $\text{Cl}^-/\text{HCO}_3^-$ (Knauf *et al.*, 2001; Jiang *et al.*, 2002; Thomson *et al.*, 2005; Xie *et al.*, 2002; Figure 2.2 A and B). Indeed, studies showed that mice with defective SLC26A6 transporters (and/or reduced expression) not only experienced hyperoxaluria but also developed renal calcium oxalate stones and showed enhanced net oxalate absorption in the ileum (Jiang *et al.*, 2006; Freel *et al.*, 2006; Garcia-Perez *et al.*, 2012; Knauf *et al.*, 2017; Khamaysi *et al.*, 2019; Jiu *et al.*, 2021). Secondly, SLC26A3 is more widely expressed in the

mammalian intestine and appears to be involved in mediating oxalate absorption in the ileum, cecum, and colon (Whittamore & Hatch, 2017; Seidler & Nikolovska, 2019; Huang *et al.*, 2020; Figure 2.2 A and B). The blood (serum) oxalate concentration was 60% lower in SLC26A3 knockout mice than those with functioning SLC26A3 (Freel *et al.*, 2013) suggesting reduced transcellular absorption. Further, the urinary oxalate excretion of SLC26A3 knockout mice was only 30% less of that measured in mice with functioning SLC26A3 (Freel *et al.*, 2013), despite the much larger decrease in plasma oxalate being filtered by the kidney. Further, the role of SLC26A3 in the kidney is thought to be reduced compared to the role in the intestine (Wedenoja *et al.*, 2008; reviewed by Freel *et al.*, 2013), corresponding to a reduced impact on renal oxalate handling. Thus, both SLC26A6 and SLC26A3 are viewed as “promising targets” when studying oxalate transport, absorption, and secretion due to the causal relationship between SLC26 (oxalate transporters), oxalate metabolism and calcium oxalate stones (especially renal), which have been observed and verified by many studies (Liu *et al.*, 2021).

Whittamore (2020) proposed that oxalate transport in seawater teleosts may involve the same molecular pathways and proteins as mammals. While there has been limited research on oxalate transport in teleosts, there are many studies which focused upon SLC26A6 and SLC26A3 expression in teleosts with regards to their role in osmoregulation. While SLC26A3 and SLC26A6 encode apical anion exchangers for which oxalate has been observed to be a substrate (Kurita *et al.*, 2008), they are also both involved in HCO_3^- secretion and Cl^- absorption along the teleost intestine (Figure 2.2; Grosell *et al.*, 2009; Gregorio *et al.*, 2013; Alper & Sharma, 2013; Seidler & Nikolovska, 2019) which aides in osmoregulation (e.g. Kurita *et al.*, 2008; Grosell *et al.*, 2009) as discussed in Chapter 1. The intestine of a seawater teleost is more alkaline than the mammalian intestine (pH ~ 8-9 (Wilson *et al.*, 2002) vs pH ~ 6-7 (Ganong, 1989)), and the source of this

increased pH appears to be generated by the intestinal epithelium through bicarbonate excretion (e.g. Grosell *et al.*, 2001; Wilson *et al.*, 1996; Grosell *et al.*, 1999; Grosell & Jensen, 1999; reviewed by Wilson *et al.*, 2002). Further, this alkaline luminal environment appears to be driven by intestinal chloride/bicarbonate anion exchangers such as SLC26A3 and SLC26A6 (Gregorio *et al.*, 2013).

In many seawater teleosts, the luminal bicarbonate concentration is highest in the anterior intestine and thus, the anterior intestine is thought to be responsible for the majority of bicarbonate secretion occurring in the intestine (Wilson *et al.*, 2002). This correlates with SLC26A6 expression which is also highest in the anterior intestine of several euryhaline teleosts (e.g. Mozambique tilapia (*Oreochromis mossambicus*, Chourasia *et al.*, 2018), Nile tilapia (*Oreochromis niloticus*, Chourasia *et al.*, 2018), gilt-head (sea) bream (*Sparus aurata*, Gregorio *et al.*, 2013), European bass (*Dicentrarchus labrax*, Alves *et al.*, 2019), and oyster toadfish (*Opsanus tau*, Grosell *et al.*, 2009)). However, in three seawater teleosts, European flounder (*Platichthys flesus*, Grosell & Jensen, 1999; Grosell *et al.*, 2001), Pacific sanddab (*Citharichthys sordidus*, Grosell *et al.*, 2001), and lemon sole (*Microstomus kitt*, Grosell *et al.*, 1999), substantial bicarbonate secretion also occurs in the mid and posterior sections of the intestine (reviewed by Wilson *et al.*, 2002), which indicates that the entire length of the teleost intestine may be involved in bicarbonate secretion. Further, while intestinal SLC26A6 expression appears to be unaffected by salinity in most euryhaline teleosts as observed in Mozambique tilapia (Chourasia *et al.*, 2018), Nile tilapia (Chourasia *et al.*, 2018), rainbow trout (*Oncorhynchus mykiss*, Genz *et al.*, 2011), and oyster toadfish (Grosell *et al.*, 2009), it was impacted by salinity in others (e.g. gilt-head (sea) bream (Gregorio *et al.*, 2013)). Together, this indicates that intestinal SLC26A6 in teleosts can be salinity dependent or independent and/or zoned along the intestine, depending upon the species.

Similar to intestinal zonation of SLC26A6, SLC26A3 expression was highest in the anterior intestine, followed by a significant decrease in the mid-intestine and further decrease in the posterior intestine as observed in European bass (Alves *et al.*, 2019). Also, Ruiz-Jarabo and colleagues (2017) found that SLC26A3 was highly expressed in both the anterior and mid-intestine of Mozambique tilapia acclimated to either freshwater or seawater. Interestingly, in Mozambique tilapia, SLC26A3 expression in the mid-intestine was observed to be approximately 4-fold higher in seawater-acclimated animals than those in freshwater (Ruiz-Jarabo *et al.*, 2017), which indicates that SLC26A3 expression may be salinity dependent like SLC26A6.

In some fish, SLC26A6 was found to have multiple isoforms (*a*, *b*, *c*) such as zebrafish (*Danio rerio*, Bayaa *et al.*, 2009) and obscure pufferfish (*Takifugu obscurus*, Kato *et al.*, 2009). Similarly, SLC26A3 was also found to have multiple isoforms (*a*, *b*) in some fish such as the Japanese eel (*Anguilla japonica*, Takei *et al.*, 2019; reviewed by Takei, 2021) and red drum (*Sciaenops ocellatus*, Esbaugh & Cutler, 2016). For the sailfin molly, there have not been any discernable studies focusing on SLC26A6 or SLC26A3 isoforms and thus, no such isoforms have yet to be reported/identified.

In the kidney, SLC26A6a likely excretes SO_4^{2-} in exchange for Cl^- but in the intestine, SLC26A6a secretes HCO_3^- in exchange for Cl^- (e.g. Watanabe & Takei, 2011; Kato *et al.*, 2009; Kurita *et al.*, 2008).). Further, Watanabe and Takei's research on seawater-acclimated Japanese eels (2011) showed that SLC26A6b and SLC26A6c are expressed in the kidneys of both freshwater and seawater eels while SLC26A6a is only expressed in the kidneys of seawater eels. In the kidneys of seawater eels, SLC26A6a and SLC26A6c are found on the apical membrane of the proximal tubule's P1 segment and SLC26A6b is located on the apical membrane of the P2 segment (Watanabe & Takei, 2011). Supporting this cellular location, SLCC26A6a was observed

to be localized in the renal proximal tubule of obscure pufferfish and this protein was involved in transporting SO_4^{2-} (Kato *et al.* 2009). In both freshwater and seawater-acclimated eels, SLC26A3 was almost exclusively expressed within the digestive tract and expression was absent within the kidneys (Watanabe and Takei, 2011). Further, studies on rats (Barmeyer *et al.*, 2007) and rabbits (Jacob *et al.*, 2002) did not detect the presence of DRA (downregulated in adenoma), which is an orthologue of mammalian SLC26A3, in the renal cortex of these animals (Freel *et al.*, 2013). However, DRA expression was detected in the human kidney (Wedenoja *et al.*, 2008; reviewed by Freel *et al.*, 2013) indicating that tissue expression is varied across species.

2.1.2.2 Microbiota

Microorganisms in the mammalian gut have been shown to play a key role in the pathogenesis and prevention of kidney stones (Mehta *et al.*, 2016). It has been shown that *O. formigenes* degrades oxalate in the gut and decreases the amount of urinary oxalate excretion in mammalian hosts (Hatch *et al.*, 2006). Further, *Lactobacillus acidophilus* is another species of oxalate-degrading bacteria that can degrade large amounts of oxalate in the mammalian gut (Miller & Dearing, 2013). Other species of oxalate-degrading bacteria such as *Lactobacillus casei* are not as efficient but can still degrade approximately a fifth as much oxalate under the same conditions (Miller & Dearing, 2013). In fish, Whittamore (2020) proposed that the teleost gut is a major excretory pathway for oxalate based on very large secretion rates (Whittamore & Hatch, 2017). While past and current literature have not examined the role of the fish gut microbiome in oxalate degradation, if the gut does act as a source of oxalate excretion, bacterial degradation would be beneficial for supporting diffusion gradients.

Unfortunately, the sailfin molly has tested negative for the presence of *O. formigenes* in its gut (Whittamore, 2020 using protocols from Allison *et al.*, 1985) and this bacterium is more

commonly found in mammalian hosts (Hatch *et al.*, 2006, 2011; reviewed by Whittamore 2020). Studies into whether *L. acidophilus* and/ or *L. casei* are the major oxalate-degrading bacterium found within the sailfin molly's gut have yet to be conducted. However, there are several studies examining the gut microbiota of fish living in freshwater or seawater (Vatsos, 2016; reviewed by Butt & Volkoff, 2019), and their findings suggest that a shift in bacterial species occurs with salinity change (Vatsos, 2016; reviewed by Butt & Volkoff, 2019; Zhang *et al.*, 2016; reviewed by Tarnecki *et al.*, 2017; Schmidt *et al.*, 2015; reviewed by Gallo *et al.*, 2020) and that members of the *Lactobacillus* genera are affected (Zhao *et al.*, 2020; Dehler *et al.*, 2017). In Atlantic salmon acclimated to either freshwater or seawater lactic acid bacteria from the order *Lactobacillales* represented 36.7% and 54.8% of all bacterial orders in the gut microbiome of freshwater and seawater-acclimated salmon respectively (Dehler *et al.*, 2017). Further, *Lactobacillus* was found to be in dominant genera in the gut microbiome of seawater-acclimated salmon, representing 95.23% of the lactic acid bacteria (Dehler *et al.*, 2017). In contrast, for freshwater-acclimated salmon, *Lactobacillus* only represented 52.28% of the lactic acid bacteria in their gut microbiome (Dehler *et al.*, 2017). Findings do not appear consistent however as bacterial species richness and diversity in the gut microbiome of farmed Chinook salmon was higher in those acclimated to freshwater than their counterparts in seawater (Zhao *et al.*, 2020). Further, *Lactobacillaceae* were in higher abundance in the intestines of freshwater-acclimated salmon, (Zhao *et al.*, 2020).

2.1.3 Hypotheses and predictions

As sailfin mollies' diet consists of quality flakes/pellets composed of vegetable matter (Hvizdak, 2014), these fish may be consuming potentially high (or excessive) amounts of oxalate through their vegetable-based diet especially compared to other carnivorous (e.g. Wels catfish (*Silurus glanis*), Verdeyroux, 2019) or omnivorous (e.g. Nile tilapia, Rakocy, 2006) teleosts.

Further, sailfin mollies are also euryhaline fish and thus, their renal handling of oxalate may also be environmentally dependent as they may rely more on intestinal excretion of oxalate in seawater (Figure 2.1). Therefore, this study aims to determine if sailfin mollies may be using their intestine as a vital pathway for excreting oxalate in an environmentally dependent manner. Since both past and current research have not studied the role of the fish gut microbiome in the degradation of oxalate, my research will address this novel idea indirectly through the use of broad-spectrum antibiotics.

Both SLC26A3 and SLC26A6 encode apical anion exchangers involved in HCO_3^- secretion and Cl^- absorption within the teleost intestine (Grosell *et al.*, 2009; Gregorio *et al.*, 2013; Alper & Sharma, 2013; Seidler & Nikolovska, 2019) but the way these transporters handle oxalate is different. SLC26A3 is involved in transporting bicarbonate ions out of the cell and transporting oxalate or chloride ions into the cell (Figure 2.2; Wang *et al.*, 2021; Sayer, 2017). While SLC26A6 is involved in transporting oxalate out of the cell in exchange for transporting chloride ions into the cell (Figure 2.2; Wang *et al.*, 2021; Sayer, 2017). Since oxalate has also been observed to be a substrate for both SLC26A3 and SLC26A6 (Kurita *et al.*, 2008), the expression patterns of these anion exchangers were also studied in the sailfin molly. The relative gene expression in the intestine and kidney of euryhaline teleosts like the sailfin molly will be key in understanding how these two osmoregulatory organs handle oxalate transport in response to changing salinities.

It was hypothesized that seawater acclimation will result in increased oxalate excretion to the intestine to either compensate for decreased renal excretion and/or aid in intestinal osmoregulation. It was also hypothesized that bacterial communities in the intestine will aid in an increased oxalate excretion in the intestine through catabolism, creating concentration gradients for transport. Since SLC26A3 is involved in net transcellular oxalate absorption, it was

hypothesized that seawater acclimation will result in downregulation of this transporter (Figure 2.1). Further, as SLC26A6 is involved in oxalate secretion, it was hypothesized that seawater acclimation will result in upregulation of this transporter (Figure 2.1). It was also predicted that SLC26A3 will be further downregulated in the antibiotic-seawater treatment while SLC26A6 will be further upregulated in the antibiotic-seawater treatment to compensate for the loss of oxalate-degrading bacteria in the intestine (Figure 2.1).

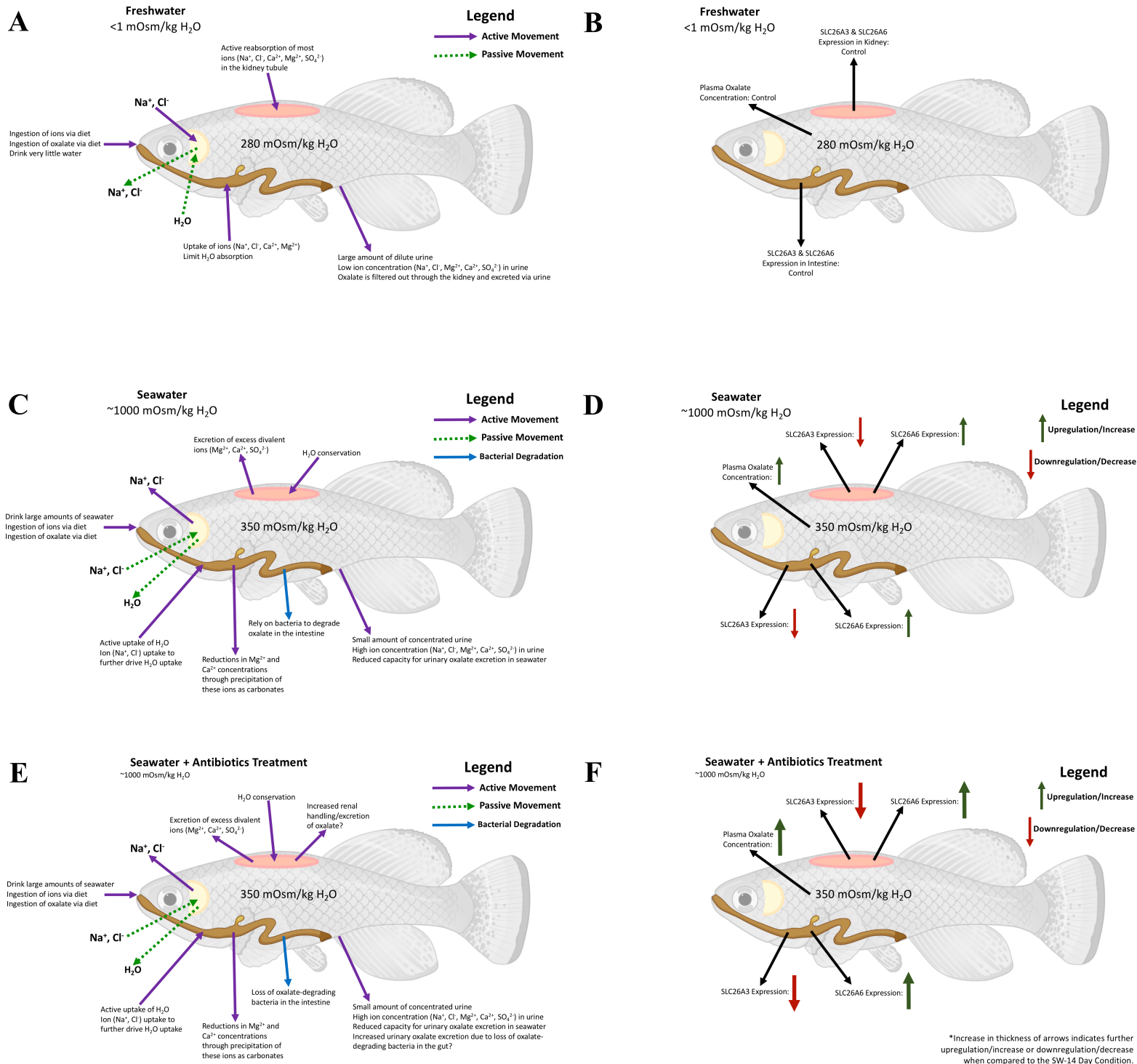


Figure 2.1 (A to F): Iono-regulation and osmoregulation strategies employed by teleosts in freshwater (A), seawater (C), and a seawater-antibiotic treatment condition (E) and the study predictions for each condition (B, D, and, F respectively). These diagrams mainly focus upon ion and water transport in the gills (beige), kidneys (pink), and intestines (brown). These schematics also illustrate the way in which oxalate might be handled within the teleost's body as they become acclimated to these three different environmental conditions. See text for more details. These illustrations were created using biorender.com.

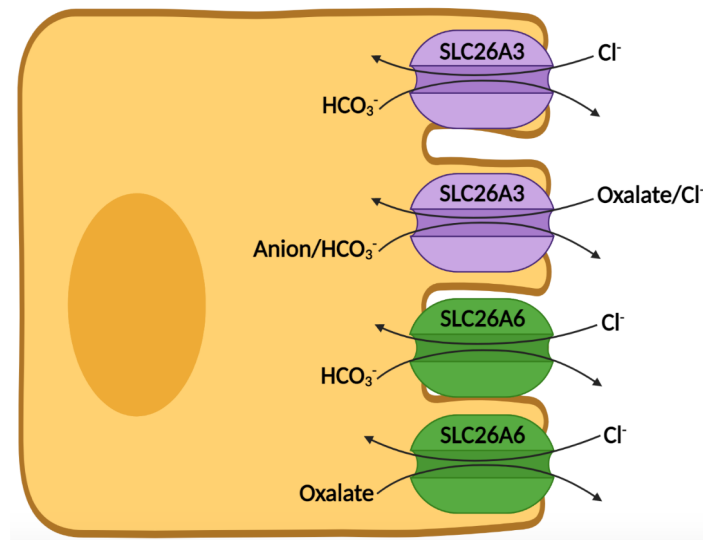
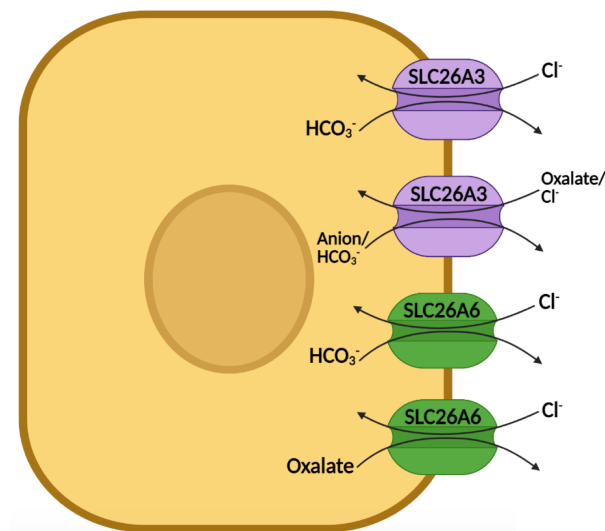
A**B**

Figure 2.2 (A and B): Predicted transport pathways of how SLC26A3 and SLC26A6 handle oxalate absorption and oxalate secretion respectively within the intestine (A) and kidney (B). Arrows indicate the transport of ions either into or out of the cell. SLC26A3 and SLC26A6 are represented as purple and green proteins respectively. These predicted transport pathways of how SLC26A3 and SLC26A6 handle oxalate (Wang *et al.*, 2021; Sayer, 2017) are for the freshwater, seawater, and antibiotic-seawater conditions but the expression levels (upregulation or downregulation) of these two transporters can change in response to the condition in which the animals are acclimated. These diagrams were created using Biorender.com.

2.2 MATERIALS AND METHODS

All experiments and animal care were carried out under an approved Animal Use Protocol from York University (2017-14 (R1)).

2.2.1 Obtaining experimental animals, housing, and fish-care

Freshwater acclimated sailfin mollies were purchased from Pet Culture (Guelph, Ontario) and transported to York University (Toronto, Ontario). Upon arrival at York University, the animals were kept in 10 gallon (37.85 L) glass aquaria (Aqueon; Franklin, Wisconsin) with approximately 15-20 fish in each tank. All animals were acclimated to dechlorinated City of Toronto freshwater and laboratory conditions for a minimum of 2 weeks prior to experimentation. Each tank contained a sponge filter with air flow and a water heater set to 24°C. Ammonia, nitrite and nitrate levels were monitored throughout acclimation and experimentation using *Freshwater Aquarium Master Test Kit* and *Saltwater Aquarium Master Test Kit* (API®; Chalfont, Pennsylvania) for freshwater and seawater conditions (including the seawater + antibiotics treatment) respectively. Water changes were conducted daily or every other day (based on ammonia, nitrite and nitrate levels) within the first 2-3 days of the fish arriving to the laboratory to minimize water ammonia levels. After the ammonia levels stabilized, water changes (50% of the tank volume) and filter cleaning were done on a weekly basis. All animals were fed 1mm pellets *Premium Fish Food – Veggie Formula* (NorthFin™; Toronto, Ontario). The animals were fed once daily at approximately 12:00 PM. Finally, the animals were subjected to a day:night cycle of 14:10 to promote a normal circadian rhythm.

2.2.2 Acclimation of animals to control and treatment conditions

After the 2-week acclimation period to the lab, fish were subjected to 1 of 4 treatments: 1) Continuation in freshwater for an additional 7 days (FW-control; 0 ppt), 2) Seawater acclimation

for 14 days (SW-14 Day; ~35 ppt), 3) Seawater acclimation for 28 days (SW-28 Day; ~ 35 ppt), and 4) Seawater acclimation with antibiotic treatment for 14 days (SW-14 Day + Antibiotics; ~35 ppt).

During seawater acclimation, sailfin mollies were acclimated to their target salinity of ~35ppt over a period of 2 days using the addition of small amounts of hypersaline water. First, hypersaline (~85ppt) water was created using 0.2 μ m filtered-dechlorinated water and Instant Ocean Sea Salt Mix (Instant Ocean, a subsidiary of Spectrum Brands; Blacksburg, Virginia). Then, over the course of 2 days, ~25% of the water was exchanged for hypersaline water to bring the salinity of the fish tanks up to ~35ppt (resulting in an increase of ~18ppt of per day). The salinity of both the hypersaline water and the fish tanks were measured in parts per thousand (ppt) using a handheld salinity refractometer (Fisher Scientific, Pittsburg, Pennsylvania). For the antibiotic treatment, four broad-spectrum antibacterial antibiotics were added to the water in an attempt to remove the bacteria in the intestine. First, 100mL of tank water was used to dissolve 0.1g of ampicillin (ampicillin, sodium salt; Millipore Corp., an affiliate of Merck KGaA, St. Louis, Missouri), 0.1g of gentamycin (gentamycin sulfate; Fisher BioReagents, Pittsburg, Pennsylvania), 10mL of penicillin (penicillin-streptomycin solution; HyClone, a subsidiary of Cytiva, Marlborough, Massachusetts) and 0.02g of kanamycin (kanamycin monosulfate; TheromScientific, Whitby, Ontario). Then, this 100mL antibiotic-seawater solution was added back into tank. The daily administration of the antibiotics was done at the time of feeding.

2.2.3 Dissections

Fish (randomly selected, both sexes) from all four conditions were sampled in the same manner, prior to feeding at approximately 11:00 AM. During euthanization, each fish was individually placed in a high-dose treatment (2.0g/L) of buffered tricane methanesulfonate (MS-

222; Syndel Canada, Nanaimo, British Columbia) dissolved in either freshwater or seawater, depending upon the condition the animal was acclimated to. The pH of this anesthetic solution used for euthanization was checked using a pH-meter (Sartorius PB-11 pH Meter, Sartorius Canada Inc., Oakville, Ontario) and was adjusted to a pH of ~7.5 (for freshwater) to 8.5 (for seawater) using NaOH (FisherScientific, Pittsburgh, Pennsylvania). Following euthanization in MS-222 a transactional cut of the spinal cord was also performed.

Sterile dissection techniques were employed for all dissections. All forceps, tweezers, and scissors used during the dissection were soaked in 70% ethanol for 15 minutes. Then, all dissection equipment (forceps, tweezers, scissors, etc ...) as well as labelled tin-foil (used to store the collected tissues) was placed under the UV fumehood (UV-PCR Workstation; Fisher Scientific, Whitby, Ontario) for a 20-minute UV sterilization. All dissections were conducted under the UV fumehood. Forceps, tweezers, scissors etc. were also decontaminated in 70% ethanol between each fish.

During dissection, the anterior intestines, posterior intestines, and whole kidneys were harvested, flash-frozen in tin-foil using dry ice and then stored at -80°C until the tissues were either used in the *Oxalate Assay Kit* (Sigma-Aldrich, Burlington, Massachusetts) or with Trizol (Invitrogen; Waltham, Massachusetts) for RNA-extractions. Blood was also collected and pooled from each of the four conditions for subsequent use in the *Oxalate Assay Kit*. The blood was collected from the initial spinal cut at the base of the head using a sterile 1mL Insulin Safety Syringe from Covidien (Dublin, Ireland). The pooled blood samples were centrifuged using a benchtop centrifuge (Fisherbrand™ Mini-Centrifuge 100-240V, 50/60Hz, Mississauga, Ontario) for ~1 minute to separate the plasma from the red blood cells. The plasma samples were stored at -20°C until they were used in the *Oxalate Assay Kit*.

To confirm the presence/absence of bacteria for the antibiotic treatments, two sentinel fish were sacrificed: one from SW-14 Day (a positive control) and one from SW-14 Day + Antibiotics. The intestines were then sterilely sampled, genomic DNA extracted, and the bacteria presence/absence confirmed using PCR.

2.2.4 Genomic DNA (gDNA) extractions for bacterial presence/absence

gDNA, including from the bacteria found in the intestine, was extracted from intestinal tissue using Qiagen's *Soil Kit Pro* (Qiagen; Germantown, Maryland). The manufacturer's protocol was used as follows. gDNA extraction was conducted following sterile technique under the UV fumehood. Any metal equipment used such as forceps, tweezers, and scissors were soaked in 70% ethanol for 15 minutes followed by UV sterilization for 20 minutes to ensure that the work area was a sterile environment. The intestinal sample was first cut into small pieces using sterile scissors. The intestinal sample was then placed into a PowerBead Pro Tube containing solution CD1 and was vortexed to disperse the sample in the buffer, dissolve humic acids, and protect the nucleic acids from degradation. The sample was homogenized horizontally at the maximum speed (on the Fisher Vortex 12-812 – Genie 2, Fisher Scientific) for 20 minutes.

Following homogenization, the PowerBead Pro Tube was centrifuged, solution CD2 (containing Inhibitor Removal Technology® (IRT)) was added to the supernatant and vortexed to mix. Solution CD2 precipitates non-DNA organic and inorganic materials such as humic acids/substances, cell debris, and proteins. Another centrifugation step was conducted to pellet the non-DNA organic and inorganic materials (humic acids, cell debris, proteins) and increase DNA yield and quality. Solution CD3, a high-salt solution was added to the supernatant, briefly vortexed to mix and then loaded onto the MB Spin Column. This allowed the DNA to bind to the silica membrane of the MB Spin Column in the presence of the high salt solution while other

contaminants passed through the filter membrane during centrifugation. Solution EA, a wash buffer was loaded onto the MB Spin Column and then centrifuged to remove proteins and other non-aqueous contaminants. Solution C5, an ethanol-based wash solution was loaded onto the MB Spin Column and centrifuged to further clean the DNA bound to silica filter membrane by removing residual salt, humic acid, and other contaminants. The empty MB Spin Column was centrifuged once again to remove any residual Solution C5 as this ethanol-based solution can interfere with downstream DNA applications such as PCR. Solution C6 (10mM Tris, lacks salt) was added to the center of the silica filter membrane of the MB Spin Column and centrifuged to facilitate the release of DNA from the membrane. This eluted gDNA was stored at -20°C until it was used in downstream applications such as PCR.

To ensure that the extraction process was sterile, a blank extraction was conducted with each set of gDNA extractions. For the blank extraction, all the above steps were performed but there was no tissue sample added.

All centrifugation steps were conducted at room temperature as per the manufacturer's protocol using a tabletop centrifuge (Eppendorf Centrifuge 5415D, Eppendorf Canada, Mississauga, Ontario).

2.2.5 Polymerase chain reaction (PCR) and gel electrophoresis

PCR on gDNA (extracted from the intestinal tissue of SW-14 Day + Antibiotics fish and SW-14 Day fish) using universal bacterial primers (8F and 533R; Sigma-Aldrich, St. Louis, Missouri) for the 16S ribosomal RNA gene (Table 2.1) was used to confirm the presence/absence of bacteria in the intestine. DreamTaq Green PCR Master Mix (2X; Thermofisher Scientific, Whitby, Ontario) was used and Thermofisher's protocol was followed for the PCR (Table 2.2). The annealing temperature was 55.5°C and 25 cycles of PCR was conducted on the extracted

gDNA. A Mastercycler® gradient thermal cycler (Eppendorf Canada Ltd.; Mississauga, Ontario) was used to run all regular PCR. A 1.5% agarose (w/v) gel was stained with ethidium bromide (50µg per 100mL gel; Thermofisher Scientific, Whitby, Ontario). Gel electrophoresis was conducted using 1X Tris-acetate-EDTA (TAE) buffer at 70V for 30 minutes. 5µL of O'RangeRuler 100bp DNA Ladder (Thermofisher Scientific, Whitby, Ontario) was run alongside 10µL of the PCR products. The gel was imaged under UV light using the MiniBIS Pro (DNR Bio-Imaging Systems; Neve Yamin, Israel). The presence of a band indicated the abundance of bacteria, whereas the absence of a band indicated little to no bacteria present (i.e. undetectable bacteria levels).

2.2.6 Oxalate assays

Using Sigma-Aldrich's *Oxalate Assay Kit*, the “free” (unprecipitated) oxalate concentrations of the anterior intestines ($\mu\text{mol mg wet tissue weight}^{-1}$), posterior intestines ($\mu\text{mol mg wet tissue weight}^{-1}$), kidneys ($\mu\text{mol mg wet tissue weight}^{-1}$), and plasma (μM) were determined. The anterior intestines, posterior intestines, and kidneys were gently homogenized using a hand-held glass homogenizer and then diluted to a colorless solution with sterile buffer prior to use in the *Oxalate Assay Kit*. The plasma samples were also diluted with sterile buffer. All dilutions were accounted for within the kit's calculations for oxalate concentration (μM). Tissue weights of the anterior intestines, posterior intestines, and kidneys were also accounted for in the calculations for oxalate concentration ($\mu\text{mol mg wet tissue weight}^{-1}$). Oxalate concentration (μM for plasma or $\mu\text{mol mg wet tissue weight}^{-1}$ for tissues) was calculated using the manufacturer's provided equation where the $\text{OD}_{\text{sample}}$, $\text{OD}_{\text{standard}}$, and OD_{blank} are the optical density measurements taken by a spectrophotometer (Synergy HT Multi-Mode Microplate Reader; BioTek; Winooski, Vermont) at 595nm for the sample, standard, and blank reactions respectively (see Equation 1).

For the equation used to calculate oxalate concentration, “SC” is the effective concentration (500 μ M) of the internal standard solution and “n” is the dilution factor (see Equation 1; Oxalate Assay Kit Protocol – MAK315, Sigma Aldrich, St. Louis, Missouri). All spectrophotometer measurements were taken at room temperature as outlined in the manufacturer’s protocol (~ 22°C).

Equation 1:

$$[\text{Oxalate}] = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{standard}} - \text{OD}_{\text{sample}}} \times \text{SC} \times n \quad (\mu\text{M})$$

2.2.7 Primer design

For this study, primers for SLC26A3 and SLC26A6 were designed de novo. According to *ensembl.org*, there is only one transcript (splice variant) of the SLC26A6 gene in the sailfin molly. However, per NCBI (National Centre for Biotechnology Information), there are two transcripts (SLC26A6 transcript variants X1 and X2) of the SLC26A6 gene in the sailfin molly. According to *ensembl.org*, there are three transcripts (splice variants) of the SLC26A3 gene in the sailfin molly (SLC26A3 transcript variants X1, X2, and X3) However, specific isoforms for both SLC26A3 and SLC26A6 in the sailfin molly have neither been identified nor studied in past research. Further, sequence similarity between the respective genes transcripts, makes individual identification difficult. Therefore, SLC26A3 and SLC26A6 were each viewed as “one” gene and the primers used in this study were designed to target all transcripts for each respective gene equally (i.e. universal primers were used for each gene).

To design the primers, the coding sequences of all transcripts of SLC26A3 and SLC26A6 for the sailfin molly were obtained from *ensembl.org* . These sequences were aligned (individually for each gene) and areas of sequence similarity were identified. NCBI’s Primer Blast online

software was then used to generate forward and reverse primers that would result in: 1) an amplicon with a length between ~200-250bp, 2) an amplicon that was similar for each gene transcript, and 3) primer sets that spanned an exon-exon junction.

Using DreamTaq Green PCR MasterMix (2X; Thermofisher Scientific, Whitby, Ontario), regular PCR was conducted on pooled sailfin cDNA (from different intestinal and kidney samples) per the protocols outlined in Tables 2 & 3. When primer sets were tested, a 25µL reaction volume was used for PCR. Initially when testing primer sets a gradient between 57°C to 62°C was used for the annealing temperatures and 40 cycles of PCR was run. A Mastercycler® gradient thermal cycler was used to run the regular PCR. A 1.5% agarose (w/v) gel was stained with ethidium bromide (50µg per 100mL gel; Thermofisher Scientific, Whitby, Ontario). Gel electrophoresis was conducted using 1X Tris-acetate-EDTA (TAE) buffer at 70V for 30 minutes. 5µL of O'RangeRuler 100bp DNA Ladder (Thermofisher Scientific, Whitby, Ontario) was run alongside 10µL of the PCR products. The gel was imaged under UV light using the MiniBIS Pro (DNR Bio-Imaging Systems; Neve Yamin, Israel). The presence of one band at the correct amplicon size for each respective primer set indicated that the correct product was amplified.

The remaining 15µL of the PCR products that resulted in one clean product at the correct amplicon size were subsequently used in the *GeneJET PCR Purification Kit* (Thermofisher Scientific, Whitby, Ontario) for DNA extraction. The manufacturer's procedure, "Protocol A: DNA purification using centrifuge" (*GeneJET PCR Purification Kit Protocol – K0701*, Thermofisher Scientific, Whitby, Ontario) was followed without any modifications. The extracted DNA was stored at -20°C until it was ready to be sent for sequencing.

The concentration (ng/µL) of the extracted DNA was determined using a spectrophotometer. According to guidelines set by the TCAG-DNA Sequencing Facility at Sick

Kids Hospital (Toronto, Ontario), the correct concentrations (ng/μL) of extracted DNA were combined with the appropriate concentration (nM) of forward and reverse primers. The samples were then submitted to the TCAG-DNA Sequencing Facility for sequencing. The sequencing results were inputted into NCBI's Nucleotide BLAST (Basic Local Alignment Search Tool) online software to confirm that each primer set was amplifying the correct gene. When the sequencing results for the SLC26A3 primer set were inputted into NCBI's Nucleotide BLAST online software, it indicated that the identity of this amplicon matched all three transcript variants of SLC26A3 identically (i.e. the primers amplified a conserved sequence between all three variants). When the sequencing results for the SLC26A6 primer set were inputted into NCBI's Nucleotide BLAST online software, it indicated that the identity of this amplicon matched both the transcript variants of SLC26A6 equally, again indicating that a conserved region of both variants was targeted. Therefore, the primer sets for SLC26A3 and SLC26A6 targeted all transcript variants for each respective gene equally. The primer sequences of SLC26A6 (product length: 233bp) and SLC26A3 (product length: 216bp) are in Table 2.1. Please see Table 3.1 in the Appendix for product sequences of SLC26A3 and SLC26A6.

All other primers using in this study were previously designed, sequenced, and validated in our lab (Bucking Lab at York University, Toronto, Ontario). Including the universal bacterial primers, 8F and 533R for the 16S rRNA gene as well as the primers for the 18S, RPL7 and RPL17 genes in the sailfin molly.

2.2.8 RNA extractions, cDNA synthesis, and quantitative PCR

RNA was extracted from the anterior intestines, posterior intestines, and kidneys using *TRIzol Reagent* according to the manufacturer's protocol with one exception. Due to the small weights of the tissues, the volumes of reagents used in the RNA extraction protocol were halved

from those published by the manufacturer for better RNA yields and RNA-quality. The RNA concentration (ng/ μ L) and quality of the samples were determined using a spectrophotometer to measure the absorbance of the samples at 260nm and 280nm. Using the absorbance values (260nm and 280nm) for the samples, the RNA concentration (ng/ μ L) and approximate quality were subsequently determined by the spectrophotometer. The RNA quality was also visually checked using an agarose gel as follows. Approximately 3-5 μ L of stock RNA (depending upon RNA concentration (ng/ μ L)) was combined with equal amount of RNA Loading Dye (2X; Thermofisher Scientific, Whitby, Ontario) and heated for 10 minutes at 70°C, cooled on ice, and ran on a 1.5% agarose (w/v) gel (stained with ethidium bromide; 50 μ g per 100mL gel; Thermofisher Scientific, Whitby, Ontario) for 30 minutes at 75V using 1X Tris-acetate-EDTA (TAE) buffer. The gels were imaged under UV light using the MiniBIS Pro (DNR Bio-Imaging Systems; Neve Yamin, Israel) and the RNA quality of the samples was visually determined. After the RNA quality was checked and the concentration (ng/ μ L) determined, cDNA was synthesized using half reactions of Promega's *cDNA Synthesis Kit*.

The three reference genes that were used for qPCR are 18S (ribosomal RNA gene), RPL7 (ribosomal protein L7 gene), and RPL17 (ribosomal protein L17 gene; Table 2.1). The 18S, RPL7, and RPL17 primers were previously sequenced and validated in our lab for this species. The optimal annealing temperature for the three reference genes was 60°C. Primers for the test genes, SLC26A6 (solute carrier family 26, member 6; product length: 233bp) and SLC26A3 (solute carrier family 26, member 3; product length: 216bp; Table 2.1) were designed and subsequently validated through sequencing. The optimal annealing temperature for both the test genes, SLC26A6 and SLC26A3 was 61°C. PowerTrack SYBR Green Master Mix (Applied Biosystems, Thermofisher Scientific, Whitby, Ontario) was used for qPCR per the manufacturer's protocol

(Table 2.3). All qPCR experiments were run for 40 cycles (Table 2.3) on the LightCycler® 96 Instrument (Roche; Mississauga, Ontario).

The raw qPCR data was analyzed using the LightCycler® 96 SW 1.1 software (Roche; Mississauga, Ontario). All samples were run in duplicate. Using the LightCycler® 96 SW 1.1 software, melt-curve analysis was conducted to ensure that there was only one product amplified in each qPCR reaction. The melt-curve analysis of all samples, including their duplicates were checked to ensure that there was only one clear melting peak and thus, only one product being amplified. The temperature at which the melting peak occurred was also verified to ensure that the correct product was being amplified in qPCR. The temperature at which the melting peak occurred for the qPCR products of SLC26A6 and SLC26A3 are 86°C and 84.5°C respectively. For both SLC26A3 and SLC26A6 primer sets, negative controls with sterile water were run as well as no reverse transcriptase (NRT) controls using RNA as the template to ensure there was no DNA contamination in the RNA preparation.

To verify that pipetting was precise, the C_q values of each sample's technical replicates were compared to ensure they were no more than 0.5 C_q apart (Nour & Pfaffl, 2020). The C_q values for each sample's technical duplicates were then averaged for subsequent use in calculating relative gene expression. Five concentrations (ng/μL) of pooled sailfin molly cDNA (from a 10-fold serial dilution) were used in the efficiency curves for both SLC26A6 and SLC26A3. Standard curves were generated using the LightCycler® 96 SW 1.1 software to determine if primer efficiencies were similar (acceptable range between 90%-110%; Taylor *et al.*, 2010; reviewed by Nour & Pfaffl, 2020) between the gene of interest (SLC26A3 or SLC26A6) and the housekeeping genes (18S, RPL7, RPL17). The relative gene expression ratios were calculated using the Livak method.

2.2.9 Statistical analyses

All oxalate assay and qPCR data were transferred into SigmaPlot Software (version 14.5, Systat Software Inc., San Jose, California). All statistical analyses were conducted using the SigmaPlot Software, which are appropriately described within their respective table and figure captions. Briefly, if needed, data was log(10)-transformed using SigmaPlot to pass normality (Shapiro-Wilk Test). Then, for normalized data sets, the Grubb's Test was run on GraphPad Prism (version 9.3, Graphpad Software, San Diego, California) to identify outliers. When three or more groups were being compared and the data was determined to be normal with a heterogeneous variance (either raw or transformed), a one-way analysis of variance (ANOVA) test, followed by Tukey *post hoc* was used. The Kruskal-Wallis one-way ANOVA on ranks, followed by Tukey *post hoc* was run on data that could not meet the conditions of normality and/or equal variance even after transformation. T-tests were used to compare two groups of data and when the conditions of normality and/or equal variance could not be met, the Mann-Whitney Rank Sum Test was used. For some data, a paired t-test was used to account for different tissues originating from the same fish and if the condition of normality could not be met, the Wilcoxon Signed Rank Test was used. Statistically significant differences were only assumed if $p < 0.05$. All graphs were created using SigmaPlot and the bars represent column means $\pm$ standard error of mean (SEM). N-values are reported in each Figure.

Table 2.1: Primer sequences, annealing temperatures, and hypervariable region (universal bacterial primers) for all the primer sets used in this study.

Gene	Primer Sequence (5' → 3')	Annealing Temperature
16S ribosomal RNA gene Universal bacterial primer set Amplified hypervariable region: V1-V3 Reference: Sun <i>et al.</i> , 2014	8F: AGAGTTTGATCCTGGCTCAG 533R: TTACCGCGGCTGCTGGCAC	55.5°C (Regular PCR)
18S ribosomal RNA gene <i>Poecilia latipinna</i> References: Murawala <i>et al.</i> , 2018; Rajaram <i>et al.</i> , 2016	Forward: GGCCGTTCTTAGTTGGTGGGA Reverse: TCAATCTCGGGTGGCTGAAC	60°C (qPCR)
Ribosomal protein L7 (RPL7) <i>Poecilia latipinna</i>	Forward: GTCTCGTATGGCTCGCAAAG Reverse: TCAGCTTGACAAACACACCG	60°C (qPCR)
Ribosomal protein L17 (RPL17) <i>Poecilia latipinna</i>	Forward: CGACCCCGAGAACCCTACTA Reverse: TTGTTGGCTTTGCGGATGTG	60°C (qPCR)
Solute carrier family 26, member 6 (SLC26A6) <i>Poecilia latipinna</i>	Forward: TCCAGTGTTTCGCCATCAGC Reverse: ATTGCTTTTCCACAGAGCGG	61°C (Regular PCR & qPCR)
Solute carrier family 26, member 3 (SLC26A3) <i>Poecilia latipinna</i>	Forward: CGGTACAAGAAAGTACAGGGGG Reverse: TCCACACCACACAATCTGGC	61°C (Regular PCR & qPCR)

Table 2.2: PCR conditions followed for regular PCR conducted with DreamTaq Green PCR MasterMix (2X; Thermofisher Scientific)

Step	Temperature	Time	Number of Cycles
Initial denaturation	95°C	2 minutes	1
Denaturation	95°C	30 seconds	25 cycles: 8F/533R For the universal bacterial primer set
Annealing	Primer Specific*	30 seconds	
Extension	72°C	1 minute	40 cycles: For 18S, SLC26A3, SLC26A3 primer sets
Final Extension	72°C	10 minutes	1
Hold	4°C	∞	—

*The annealing temperature varies for each of the different primer sets (see Table 2.1).

Table 2.3: PCR conditions followed for qPCR conducted with PowerTrack SYBR Green Master Mix (Applied Biosystems, Thermofisher Scientific)

Step	Temperature	Duration	Cycles
Enzyme activation	95°C	2 minutes	1
Denature	95°C	15 seconds	40
Anneal/extend	60°C for 18S, RPL7, RPL17 61°C for SLC26A3, SLC26A6	60 seconds	

2.3 RESULTS

2.3.1 Salinity Acclimation: Short-term (14-day) and long-term (28-day) salinity exposures

When the plasma oxalate concentrations were examined, the SW-14 Day condition was significantly higher than the FW condition (increasing 1.32-fold from $151.43 \pm 7.60 \mu\text{M}$ (N=7) to $200.37 \pm 6.24 \mu\text{M}$ (N=7); $p < 0.001$; Figure 2.3A). Following 28-days of acclimation to seawater however, the oxalate concentration in the plasma had returned to control (FW) levels ($144.88 \pm 6.36 \mu\text{M}$ (N=7); Figure 2.3A). Interestingly, there were no observed differences between the oxalate concentrations of the kidneys from the FW condition ($48.74 \pm 16.03 \mu\text{mol mg wet tissue weight}^{-1}$ (N=7)), SW-14 Day condition ($44.27 \pm 5.74 \mu\text{mol mg wet tissue weight}^{-1}$ (N=7)), and SW-28 Day condition ($27.95 \pm 8.55 \mu\text{mol mg wet tissue weight}^{-1}$ (N=7)), averaging $\sim 40 \mu\text{mol mg wet tissue weight}^{-1}$ ($p = 0.116$; Figure 2.3B) across conditions. In contrast, the oxalate concentration in the anterior intestine of animals acclimated to the SW-14 Day condition more than doubled to $15.10 \pm 2.05 \mu\text{mol mg wet tissue weight}^{-1}$ (N=10) from $6.25 \pm 1.23 \mu\text{mol mg wet tissue weight}^{-1}$ (N=10) ($p = 0.003$; Figure 2.3C) paralleling the observed plasma increase. Further, the oxalate concentration of the anterior intestine in the SW-28 Day condition ($13.85 \pm 3.48 \mu\text{mol mg wet tissue weight}^{-1}$ (N=10)) was considered to be intermediate to both the FW and SW-14 Day conditions ($p > 0.05$; Figure 2.3C). For the posterior intestine, seawater acclimation gradually lowered the oxalate concentration from that in freshwater by $\sim 63\%$ from $17.03 \pm 6.42 \mu\text{mol mg wet tissue weight}^{-1}$ (N=10) to $6.24 \pm 0.95 \mu\text{mol mg wet tissue weight}^{-1}$ (N=10; $p = 0.045$) by 28 days post-acclimation (Figure 2.3D).

Surprisingly, SLC26A3 expression was undetectable in the kidneys (Figures 3.2A & 3.2B in the Appendix). However, during both short-term and long-term salinity exposure, the impact of salinity on SLC26A3 expression was similar in both the anterior and posterior sections of the

intestine (Figure 2.4). Specifically, SLC26A3 expression in the anterior intestine for the SW-14 Day condition ($14.67 \pm 2.95\%$ (N=10)) and SW-28 Day condition ($7.88 \pm 1.30\%$ (N=10)) were 85% and 92% lower than the FW condition respectively ($p < 0.001$; Figure 2.4A). Further, SLC26A3 expression in the posterior intestine for the SW-14 Day condition ($4.94 \pm 0.75\%$ (N=10)) and SW-28 Day condition ($4.66 \pm 0.72\%$ (N=10)) were both ~95% lower than the FW condition ($p < 0.001$; Figure 2.4B). There was no difference in SLC26A3 expression between the SW-14 Day and SW-28 Day conditions in both the anterior and posterior intestines ($p > 0.05$; Figure 2.4).

SLC26A6 expression in the kidney of the SW-14 Day condition ($334.29 \pm 69.55\%$ (N=7)) increased 3.3-fold from the FW condition and remained elevated in the SW-28 Day condition ($706.73 \pm 175.69\%$ (N=7); $p < 0.001$; Figure 2.5A). Additionally, during seawater acclimation SLC26A6 expression in the anterior intestine increased by 5.3-fold and 5.8-fold in the SW-14 Day condition ($530.63 \pm 68.44\%$ (N=10)) and SW-28 Day condition ($577.14 \pm 63.96\%$ (N=10)) respectively when compared to the anterior intestine of the FW condition ($p < 0.001$; Figure 2.5B). As with SLC26A3, when SLC26A6 expression in the anterior intestine of the SW-14 Day and SW-28 Day conditions were compared, there was no statistically significant difference ($p > 0.05$; Figure 2.5B). In comparison to the anterior intestine, SLC26A6 expression in the posterior intestine of the SW-14 Day condition ($311.18 \pm 34.68\%$ (N=10)) was 3.11-fold higher than the FW condition ($p = 0.002$; Figure 2.5C). However, in contrast SLC26A6 expression in the posterior intestine of the SW-28 Day condition ($194.12 \pm 18.63\%$ (N=10)) was statistically the same as both the FW and SW-14 Day conditions ($p > 0.05$; Figure 2.5C).

2.3.2 Antibiotic Treatment: SW-14 Day + Antibiotics exposure

2.3.2.1 Effectiveness of antibiotic treatment using PCR and gel electrophoresis

After it was confirmed that the 8F/533R primers did not yield a product in the antibiotic treated fish while a product was seen in untreated fish (Figure 3.1 in the Appendix), the remainder of the SW-14 Day + Antibiotics fish were used in subsequent analyses.

2.3.2.2 Results of the SW-14 Day + Antibiotics exposure

Following antibiotic treatment, the plasma oxalate increased 1.26-fold from 200.37 ± 6.24 μM (N=7) in SW-14 Day to 252.39 ± 10.33 μM (N=7) in SW-14 Day + Antibiotics ($p=0.002$; Figure 2.6A). However, when the oxalate concentration of the kidney in the SW-14 Day condition (44.27 ± 5.74 $\mu\text{mol mg wet tissue weight}^{-1}$ (N=7)) was directly compared to the SW-14 Day + Antibiotics condition (67.60 ± 14.15 $\mu\text{mol mg wet tissue weight}^{-1}$ (N=7)), there was again no statistically significant difference ($p=0.412$; Figure 2.6B). In contrast, the oxalate concentration in the anterior intestine of the SW-14 Day + Antibiotics condition (7.06 ± 0.85 $\mu\text{mol mg wet tissue weight}^{-1}$ (N=10)) was less than half of the SW-14 Day condition (15.10 ± 2.05 $\mu\text{mol mg wet tissue weight}^{-1}$ (N=10)) ($p=0.002$; Figure 2.6C). Further, the posterior intestine of the SW-14 Day + Antibiotics condition had an oxalate concentration of 18.22 ± 2.30 $\mu\text{mol mg wet tissue weight}^{-1}$ (N=10), approximately double the oxalate concentration of the SW-14 Day condition (9.13 ± 1.17 $\mu\text{mol mg wet tissue weight}^{-1}$ (N=10); $p=0.008$; Figure 2.6D).

There were similar impacts observed in SLC26A3 expression levels in both the anterior and posterior intestines for the SW-14 Day + Antibiotics treatment (Figure 2.7). When SLC26A3 expression was directly compared in the anterior intestines of the SW-14 Day condition ($100.00 \pm 20.07\%$ (N=10)) and SW-14 Day + Antibiotics condition ($34.66 \pm 7.28\%$ (N=10)), the SW-14 Day

+ Antibiotics condition displayed a 65% reduction in SLC26A3 gene expression ($p=0.010$; Figure 2.7A). Similarly, when SLC26A3 expression was directly compared in the posterior intestines of the SW-14 Day condition ($100.00 \pm 15.20\%$ ($N=10$)) and SW-14 Day + Antibiotics condition ($56.39 \pm 8.61\%$ ($N=10$)), the SW-14 Day + Antibiotics condition was 44% lower ($p=0.029$; Figure 2.7B).

When expression of SLC26A6 in the kidney of SW-14 Day + Antibiotics condition ($1496.40 \pm 212.22\%$ ($N=7$)) fish was compared directly to those in the SW-14 Day condition ($100.00 \pm 20.80\%$ ($N=7$)), antibiotics significantly increased SLC26A6 expression by nearly 15-fold ($p<0.001$; Figure 2.8A). However, when the intestine was examined, the impacts of antibiotics on SLC26A6 expression were similar to the impacts on SLC26A3. Specifically, the SLC26A6 expression in the anterior intestine of the SW-14 Day + Antibiotics condition ($29.73 \pm 6.56\%$ ($N=10$)) was 70% lower than the SW-14 Day condition ($p<0.001$; Figure 2.8B). Similarly, SLC26A6 expression in the posterior intestines was 84 % lower in the SW-14 Day + Antibiotics condition ($15.69 \pm 1.33\%$ ($N=10$)) than the SW-14 Day condition ($p<0.001$; Figure 2.8C).

2.3.3 Zonation: Comparing the anterior and posterior intestines across all conditions

The oxalate concentrations of the anterior and posterior sections of intestine were found to be significantly different ($p<0.05$; Figure 2.9) regardless of treatment. Specifically, in the FW condition oxalate concentration increased along the intestine, rising three-fold to $17.03 \pm 6.42 \mu\text{mol mg wet tissue weight}^{-1}$ ($N=10$) in the posterior intestine from $6.25 \pm 1.23 \mu\text{mol mg wet tissue weight}^{-1}$ ($N=10$; $p=0.002$) in the anterior intestine (Figure 2.9A). In contrast, seawater acclimation resulted in decreasing oxalate concentrations along the intestine regardless of the length of salinity acclimation. In particular, in both the SW-14 Day and SW-28 Day conditions, the oxalate concentration of the anterior intestine was more than double the concentration in the posterior

intestine ($p=0.016$ and $p=0.010$ respectively; Figures 2.9B & 2.9C). For the SW-14 Day condition, the oxalate concentrations of the anterior and posterior intestines were $15.10 \pm 2.05 \mu\text{mol mg wet tissue weight}^{-1}$ ($N=10$) and $9.13 \pm 1.17 \mu\text{mol mg wet tissue weight}^{-1}$ ($N=10$) respectively (Figure 2.9B). Similarly, for the SW-28 Day condition, the oxalate concentrations of the anterior and posterior intestines were $13.85 \pm 3.48 \mu\text{mol mg wet tissue weight}^{-1}$ ($N=10$) and $6.24 \pm 0.95 \mu\text{mol mg wet tissue weight}^{-1}$ ($N=10$) respectively (Figure 2.9C). Interestingly, exposure to antibiotics in seawater acclimated animals resulted in a similar zonation as was observed in FW, with increasing concentrations along the intestine. Specifically, in the SW-14 Day + Antibiotics condition, the posterior intestine had an oxalate concentration of $18.22 \pm 2.30 \mu\text{mol mg wet tissue weight}^{-1}$ ($N=10$), which was more than double the oxalate concentration of the anterior intestine, which was only $7.06 \pm 0.85 \mu\text{mol mg wet tissue weight}^{-1}$ ($N=10$; $p=0.001$; Figure 2.9D).

Zonation of SLC26A3 expression along the intestine was similar regardless of treatment (FW, SW-14 Day, SW-28 Day, SW-14 Day + Antibiotics). Specifically, SLC26A3 expression was reduced in the posterior intestines of the FW condition (Figure 2.10A) indicating a decrease in expression along the intestine. SLC26A3 expression similarly decreased along the intestine when fish were acclimated to seawater for 14 days (Figure 2.10B) and 28 days (Figure 2.10C). Finally, antibiotic exposure did not alter the zonation of expression with the expression decreasing nearly 75% along the intestine (Figure 2.10D) as in other conditions.

In contrast to the intestinal zonation of SLC26A3, when SLC26A6 expression was directly compared between the anterior ($100.00 \pm 37.46\%$ ($N=10$)) and posterior ($50.00 \pm 17.31\%$ ($N=10$)) sections of the intestine within the FW condition, there was no statistically significant difference in expression levels ($p=0.209$; Figure 2.11A). However, zonation along the intestine of the remaining treatments indicated a similar decreasing pattern along the intestine as observed with

SLC26A3. Specifically, SLC26A6 expression in the anterior and posterior intestines of the SW-14 Day condition were $100.00 \pm 12.90\%$ (N=10) and $29.32 \pm 3.27\%$ (N=10) respectively (Figure 2.11B) while SLC26A6 expression in the anterior and posterior intestines of the SW28-Day condition were $100.00 \pm 11.08\%$ (N=10) and 16.82 ± 1.61 (N=10) respectively (Figure 2.11C). Finally, SLC26A6 expression in the anterior and posterior intestines of the SW-14 Day + Antibiotics condition were $100.00 \pm 22.08\%$ (N=10) and $15.47 \pm 1.31\%$ (N=10) respectively (Figure 2.11D).

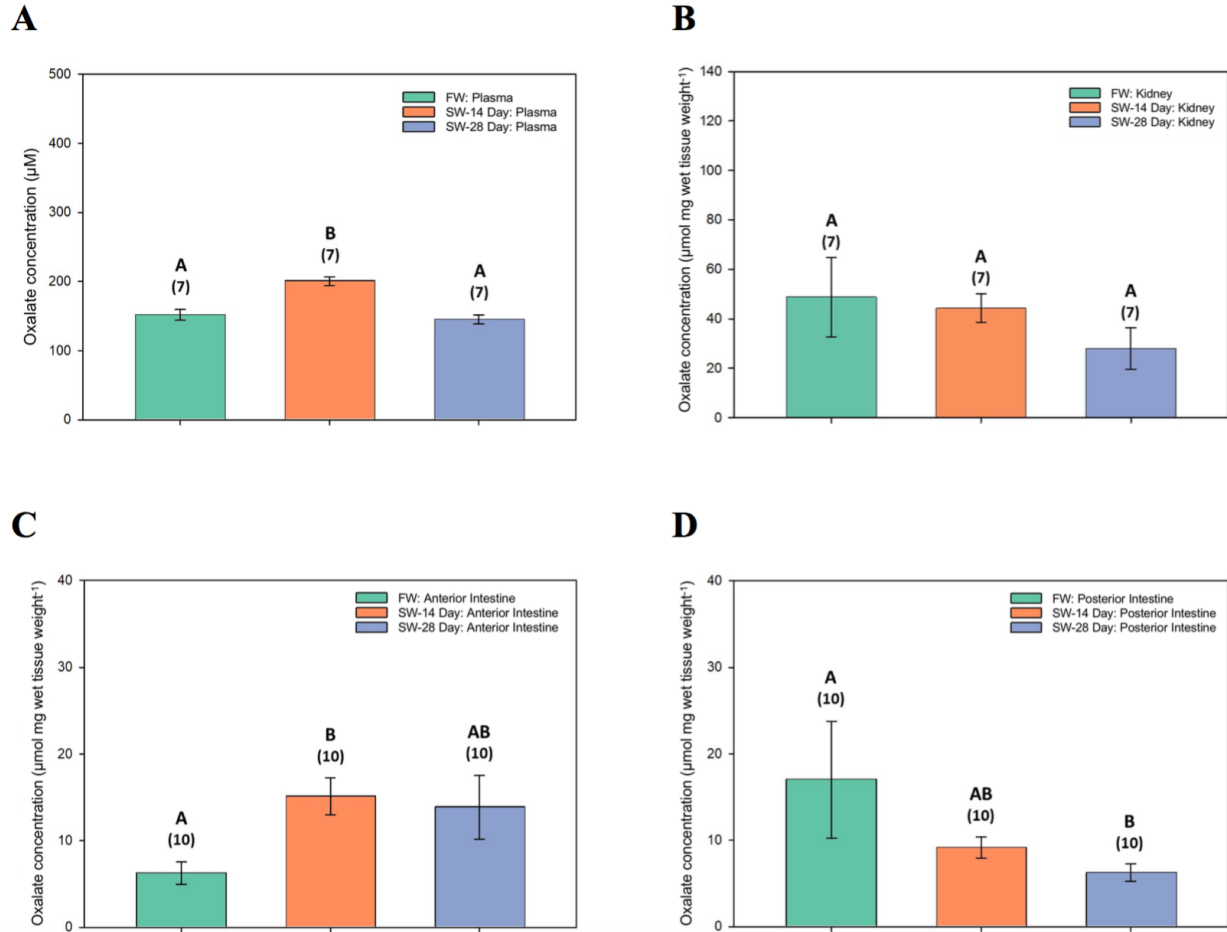


Figure 2.3 (A to D): Comparing oxalate concentrations of the plasma (A; μM), kidneys (B; $\mu\text{mol mg wet tissue weight}^{-1}$), anterior intestines (C; $\mu\text{mol mg wet tissue weight}^{-1}$), and posterior intestines (D; $\mu\text{mol mg wet tissue weight}^{-1}$) across the FW, SW-14 Day, and SW-28 Day conditions. The bars represent column means $\pm$ SEM and the numbers in the brackets represent n-values. For the plasma, each n-value was a pooled sample of 5 fish. Within each panel, bars that share letters are not statistically different. As not all data passed parametric parameters the following statistical tests were used: Figure 2.3A: A one-way ANOVA with Tukey *post hoc* ($p < 0.001$); Figure 2.3B: A one-way ANOVA with Tukey *post hoc* on $\log(10)$ -transformed data ($p = 0.116$); Figure 2.3C: ANOVA-on ranks with Tukey *post hoc* ($p = 0.003$); Figure 2.3D: A one-way ANOVA with Tukey *post hoc* on $\log(10)$ -transformed data ($p = 0.045$).

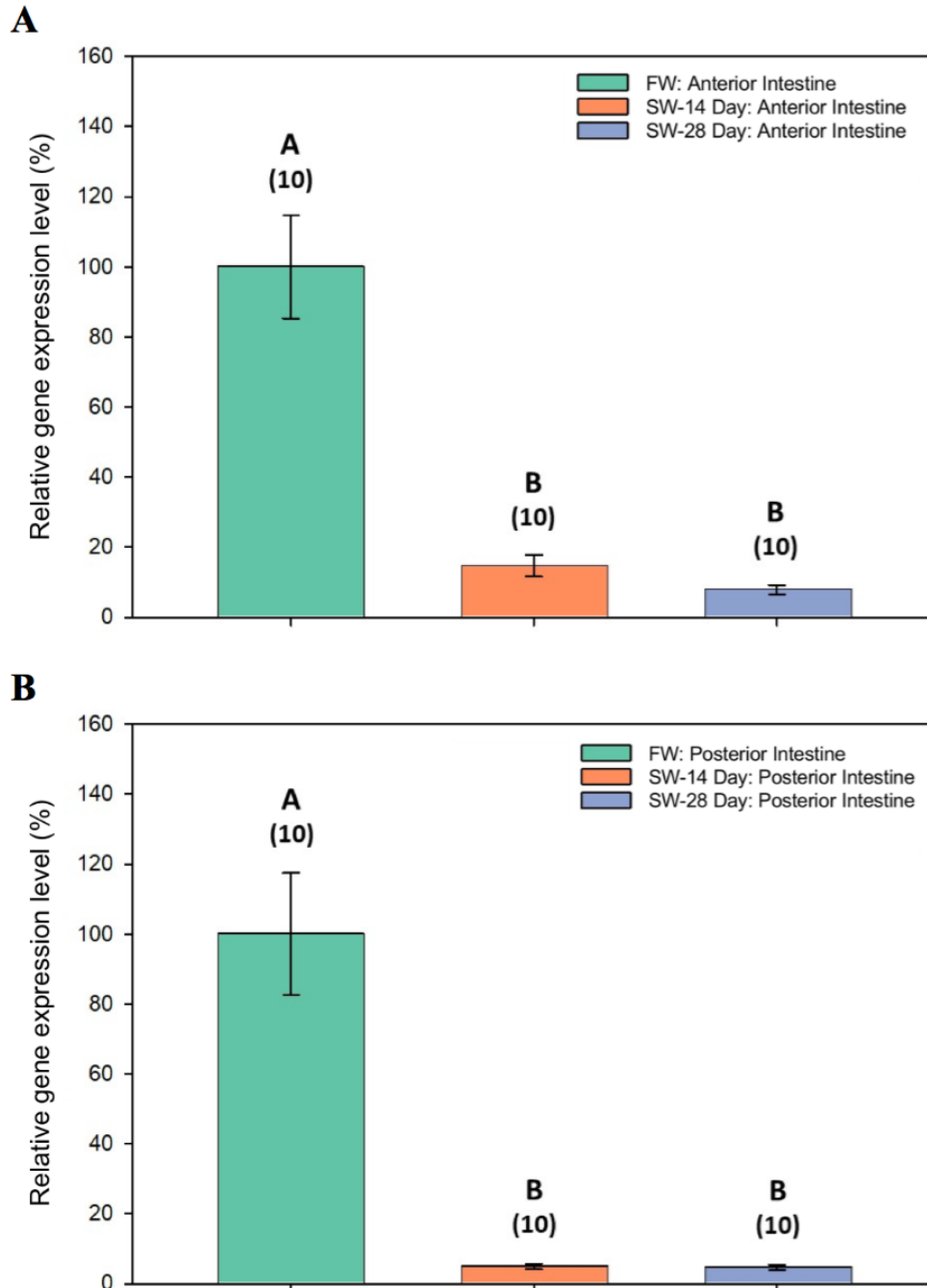


Figure 2.4 (A and B): Comparing relative SLC26A3 gene expression in the anterior (A; %) and posterior intestines (B; %) across the FW, SW-14 Day, and SW-28 Day conditions. The relative gene expression levels are expressed as percentages with the control (FW) condition set to 100%. The bars represent column means $\pm$ SEM and the numbers in the brackets represent n-values. Within each panel, bars that share letters are not statistically different. As not all data passed parametric parameters the following statistical tests were used: Figure 2.4A: An ANOVA-on ranks with Tukey *post hoc* ($p < 0.001$); Figure 2.4B: A one-way ANOVA with Tukey *post hoc* on $\log(10)$ -transformed data ($p < 0.001$).

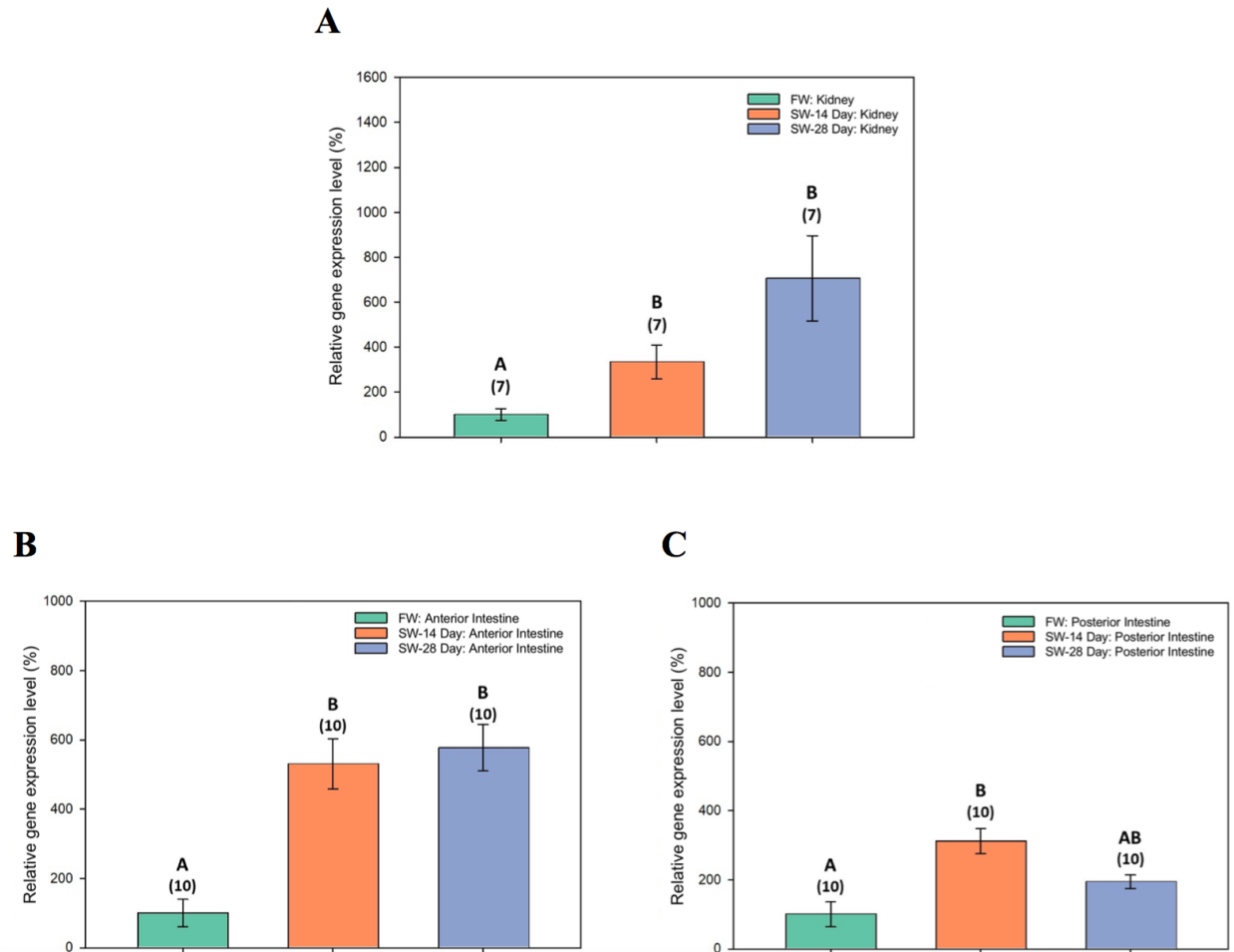


Figure 2.5 (A to C): Comparing relative SLC26A6 gene expression in the kidney (A; %), anterior intestine (B; %), and posterior intestine (C; %) across the FW, SW-14 Day, and SW-28 Day conditions. The relative gene expression levels are expressed as percentages with the control (FW) condition set to 100%. The bars represent column means $\pm$ SEM and the numbers in the brackets represent n-values. Within each panel, bars that share letters are not statistically different. As not all data passed parametric parameters the following statistical tests were used: Figure 2.5A: A one-way ANOVA with Tukey *post hoc* on log(10)-transformed data ($p < 0.001$); Figure 2.5B: A one-way ANOVA with Tukey *post hoc* ($p < 0.001$); Figure 2.5C: An ANOVA-on ranks with Tukey *post hoc* ($p = 0.002$).

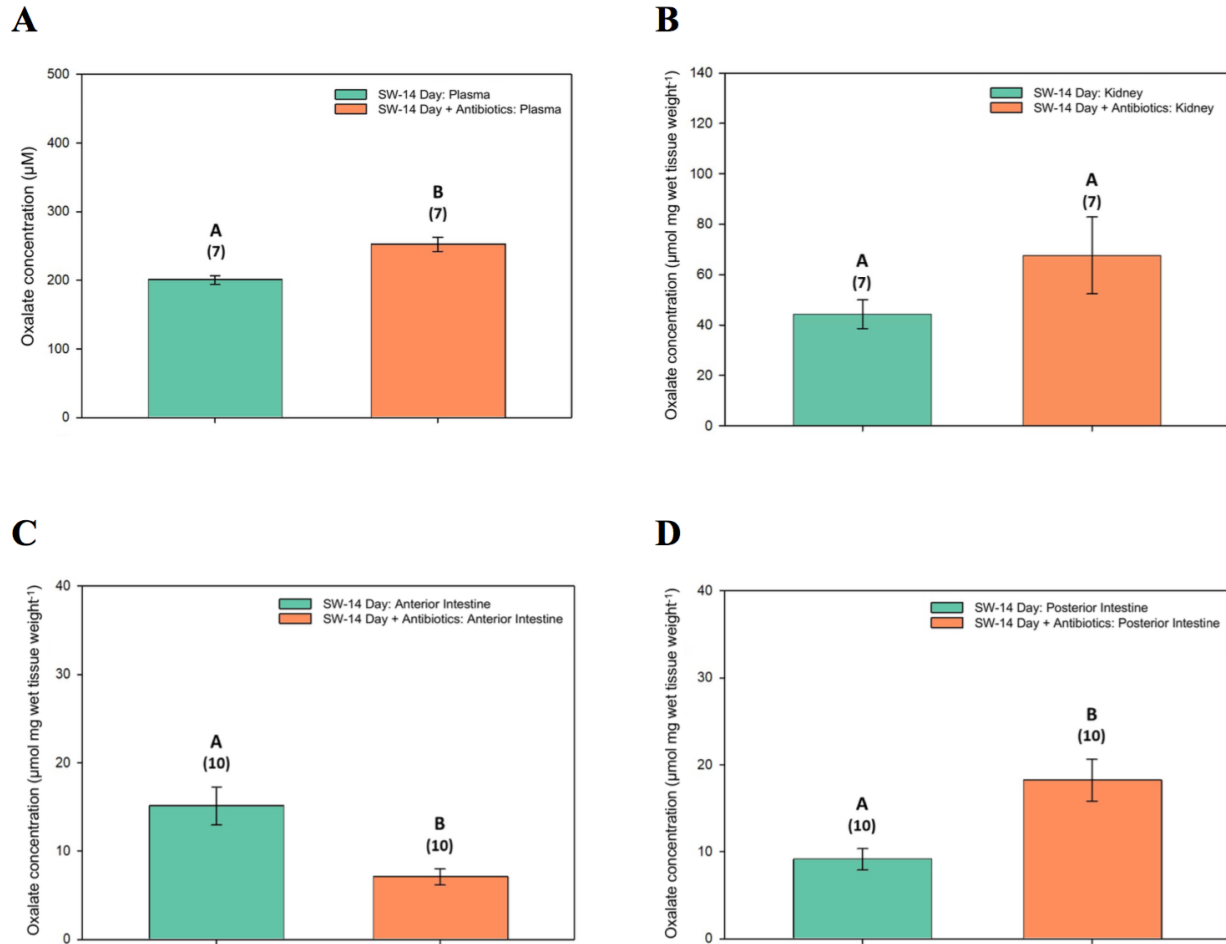


Figure 2.6 (A to D): Comparing oxalate concentrations between the SW-14 Day condition and the SW-14 Day + Antibiotics condition in the plasma (A; μM), kidney (B; $\mu\text{mol mg wet tissue weight}^{-1}$), anterior intestine (C; $\mu\text{mol mg wet tissue weight}^{-1}$), and posterior intestine (D; $\mu\text{mol mg wet tissue weight}^{-1}$). The bars represent column means $\pm$ SEM and the numbers in the brackets represent n-values. For the plasma, each n-value was a pooled sample of 5 fish. Within each panel, bars that share letters are not statistically different. As not all data passed parametric parameters the following statistical tests were used: Figure 2.6A: A two-tailed t-test ($p=0.002$); Figure 2.6B: A two-tailed t-test on $\log(10)$ transformed data ($p=0.412$); Figure 2.6C: A Mann-Whitney Rank Sum Test ($p=0.002$); Figure 2.6D: A Mann-Whitney Rank Sum Test ($p=0.008$).

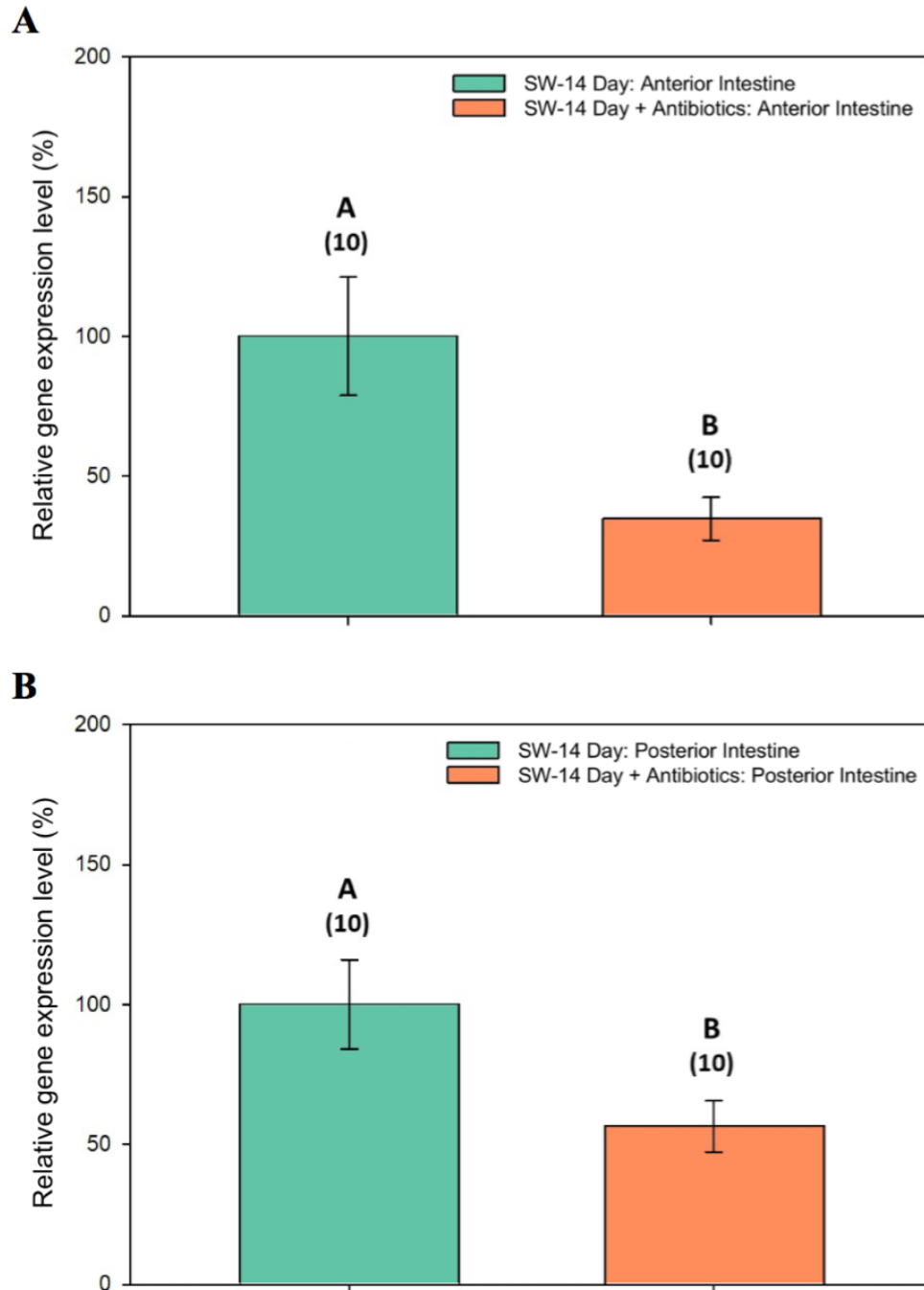


Figure 2.7 (A and B): Comparing relative SLC26A3 gene expression levels between the SW-14 Day condition and the SW-14 Day + Antibiotics condition in the anterior intestines (A; %) and posterior intestines (B; %). The relative gene expression levels are expressed as percentages with the SW-14 Day condition set to 100%. The bars represent column means $\pm$ SEM and the numbers in the brackets represent n-values. Within each panel, bars that share letters are not statistically different. As not all data passed parametric parameters the following statistical tests were used: Figure 2.7A: A Mann-Whitney Rank Sum Test ($p=0.010$); Figure 2.7B: A two-tailed t-test ($p=0.029$).

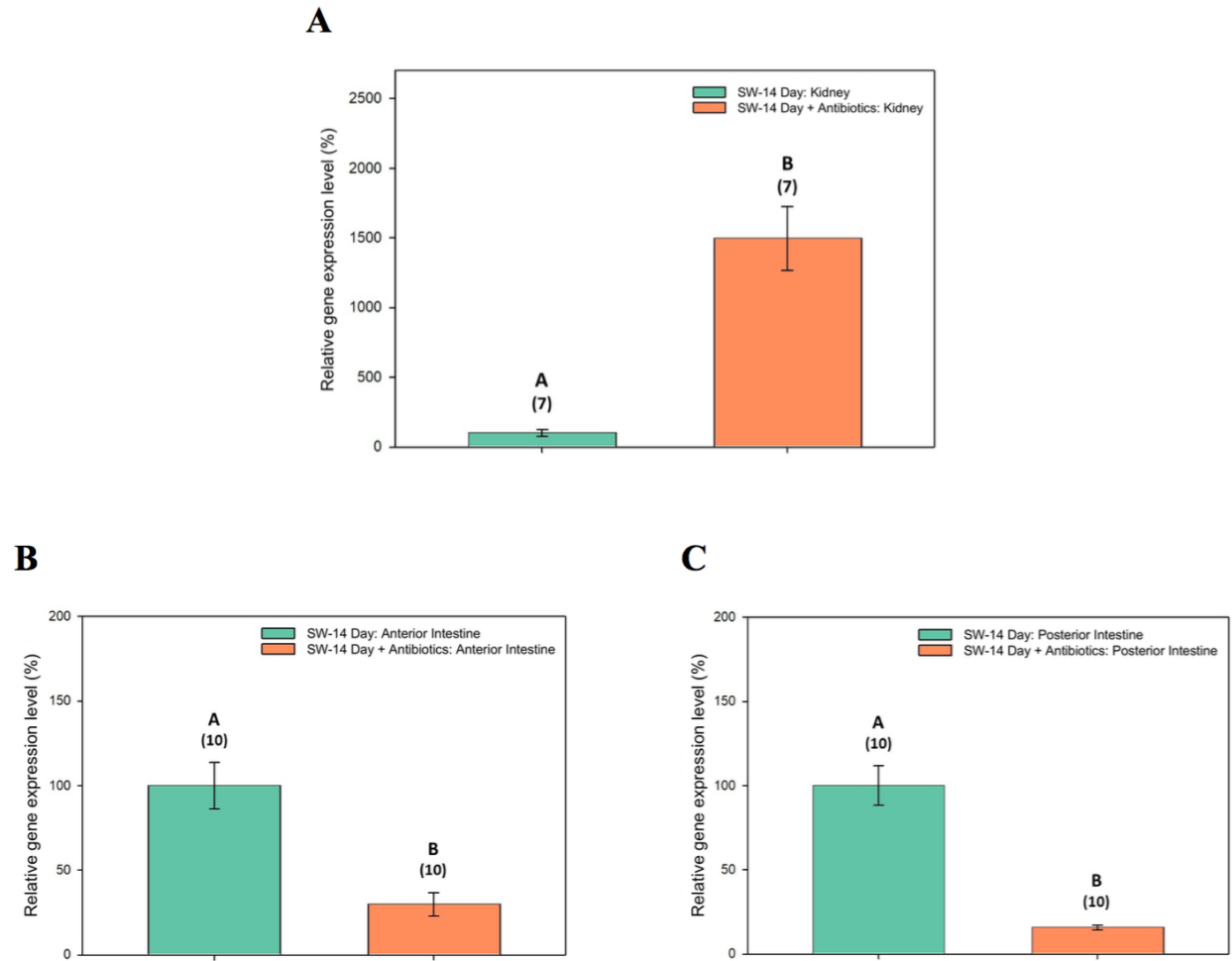


Figure 2.8 (A to C): Comparing relative SLC26A6 gene expression levels between the SW-14 Day condition and the SW-14 Day + Antibiotics condition in the kidneys (A; %), anterior intestines (B; %) and posterior intestines (C; %). The relative gene expression levels are expressed as percentages with the SW-14 Day condition set to 100%. The bars represent column means $\pm$ SEM and the numbers in the brackets represent n-values. Within each panel, bars that share letters are not statistically different. As not all data passed parametric parameters the following statistical tests were used: Figure 2.8A: A Mann-Whitney Rank Sum Test ($p < 0.001$); Figure 2.8B: A two-tailed t-test ($p < 0.001$); Figure 2.8C: A Mann-Whitney Rank Sum Test ($p < 0.001$).

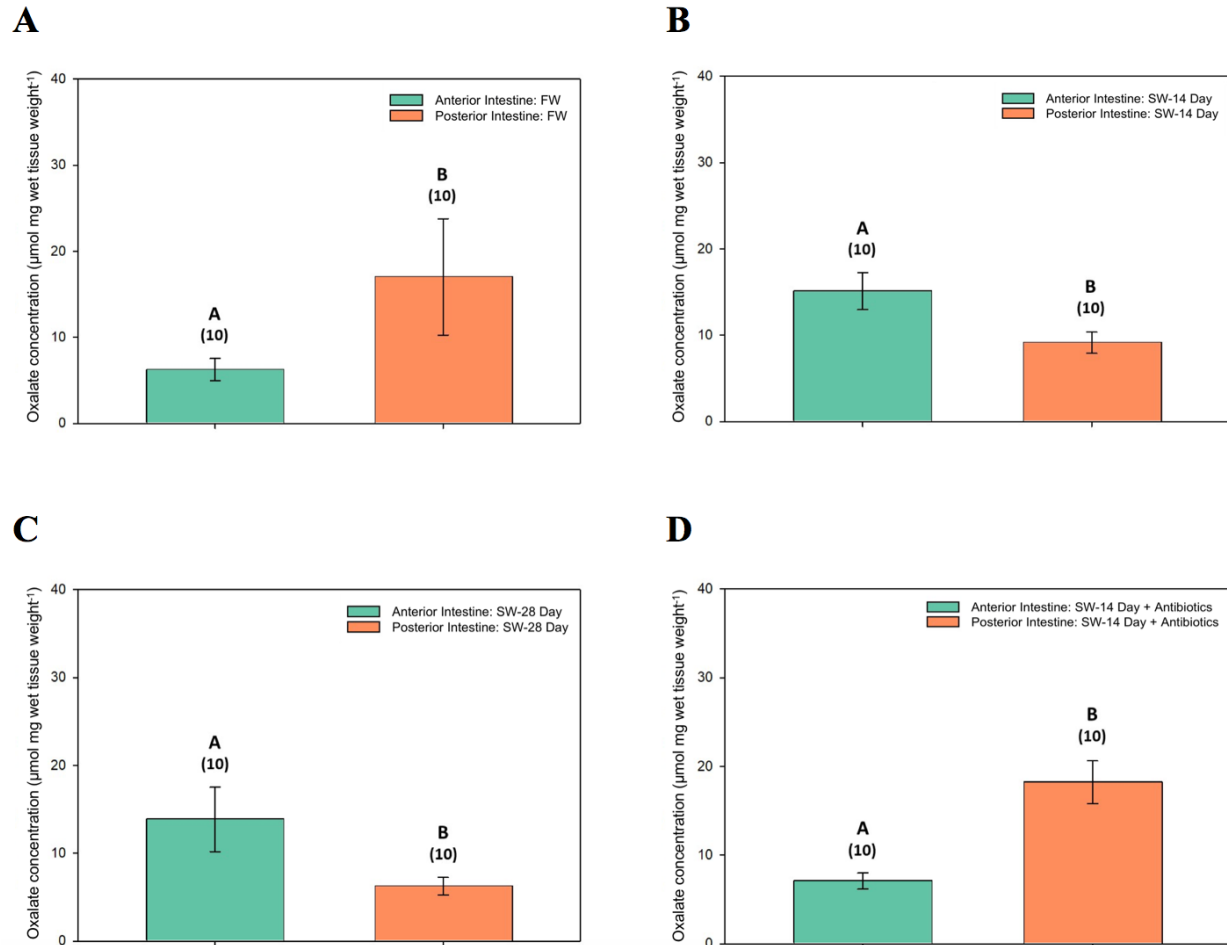


Figure 2.9 (A to D): Comparing oxalate concentrations between the anterior and posterior intestines of the FW (A; $\mu\text{mol mg wet tissue weight}^{-1}$), SW-14 Day (B; $\mu\text{mol mg wet tissue weight}^{-1}$), SW-28 Day (C; $\mu\text{mol mg wet tissue weight}^{-1}$), and the SW-14 Day + Antibiotics conditions (D; $\mu\text{mol mg wet tissue weight}^{-1}$). The bars represent column means $\pm$ SEM and the numbers in the brackets represent n-values. Within each panel, bars that share letters are not statistically different. As not all data passed parametric parameters the following statistical tests were used: Figure 2.9A: A Mann-Whitney Rank Sum Test ($p=0.034$) and a Wilcoxon Signed Rank Test ($p=0.002$); Figure 2.9B: A paired, two-tailed t-test ($p=0.016$); Figure 2.9C: A Mann-Whitney Rank Sum Test ($p=0.007$) and a Wilcoxon Signed Rank Test ($p=0.010$); Figure 2.9D: A paired, two-tailed t-test ($p=0.001$).

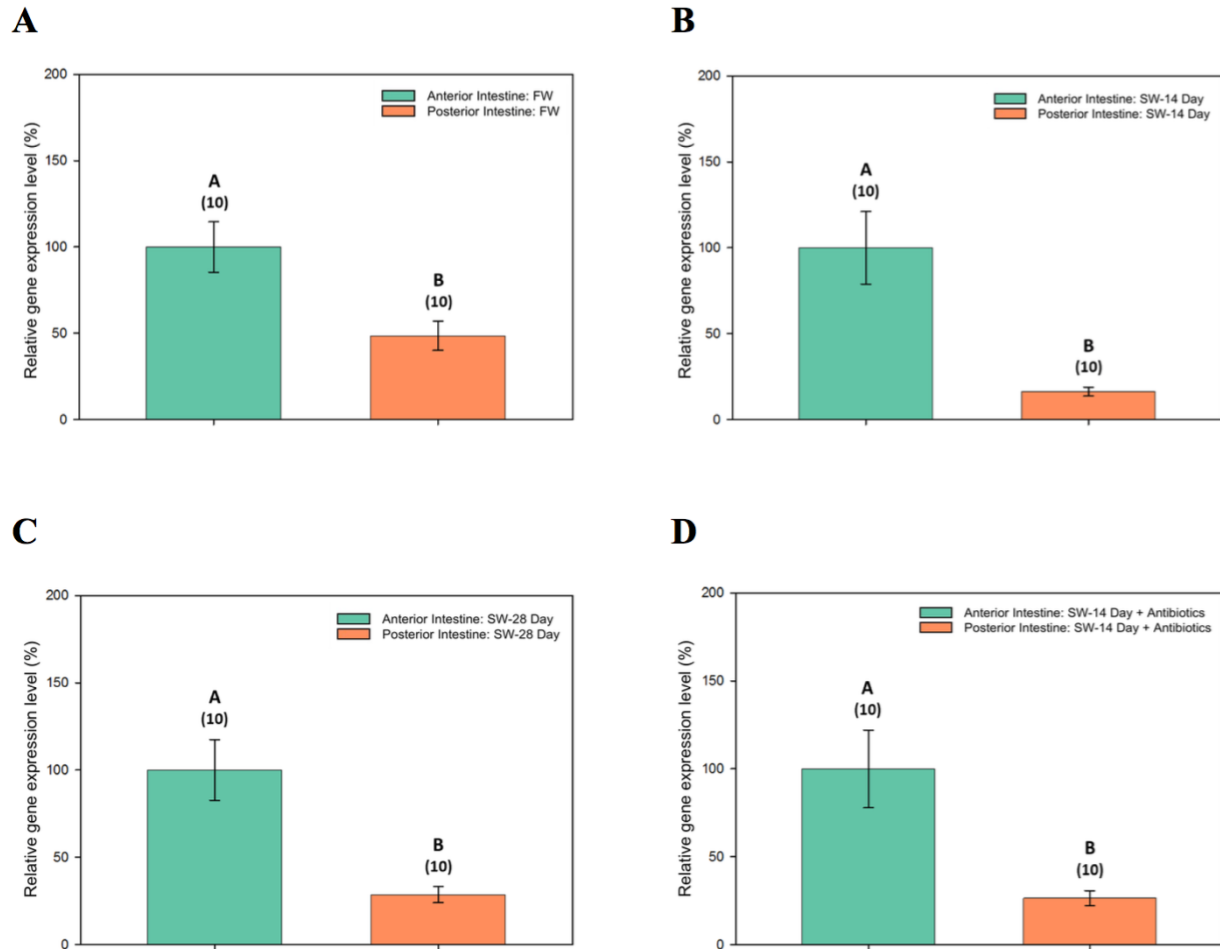


Figure 2.10 (A to D): Comparing relative SLC26A3 gene expression levels between the anterior and posterior intestines of the FW (A; %), SW-14 Day (B; %), SW-28 Day (C; %), and the SW-14 Day + Antibiotics (D; %) conditions. The relative gene expression levels are expressed as percentages with the anterior intestine in each condition set to 100%. The bars represent column means $\pm$ SEM and the numbers in the brackets represent n-values. Within each panel, bars that share letters are not statistically different. All data passed parametric parameters and the following statistical tests were used: Figure 2.10A: A paired, two-tailed t-test ($p=0.003$); Figure 2.10B: A paired, two-tailed t-test on $\log(10)$ -transformed data ($p<0.001$); Figure 2.10C: A paired, two-tailed t-test ($p=0.002$); Figure 2.10D: A paired, two-tailed t-test ($p=0.006$).

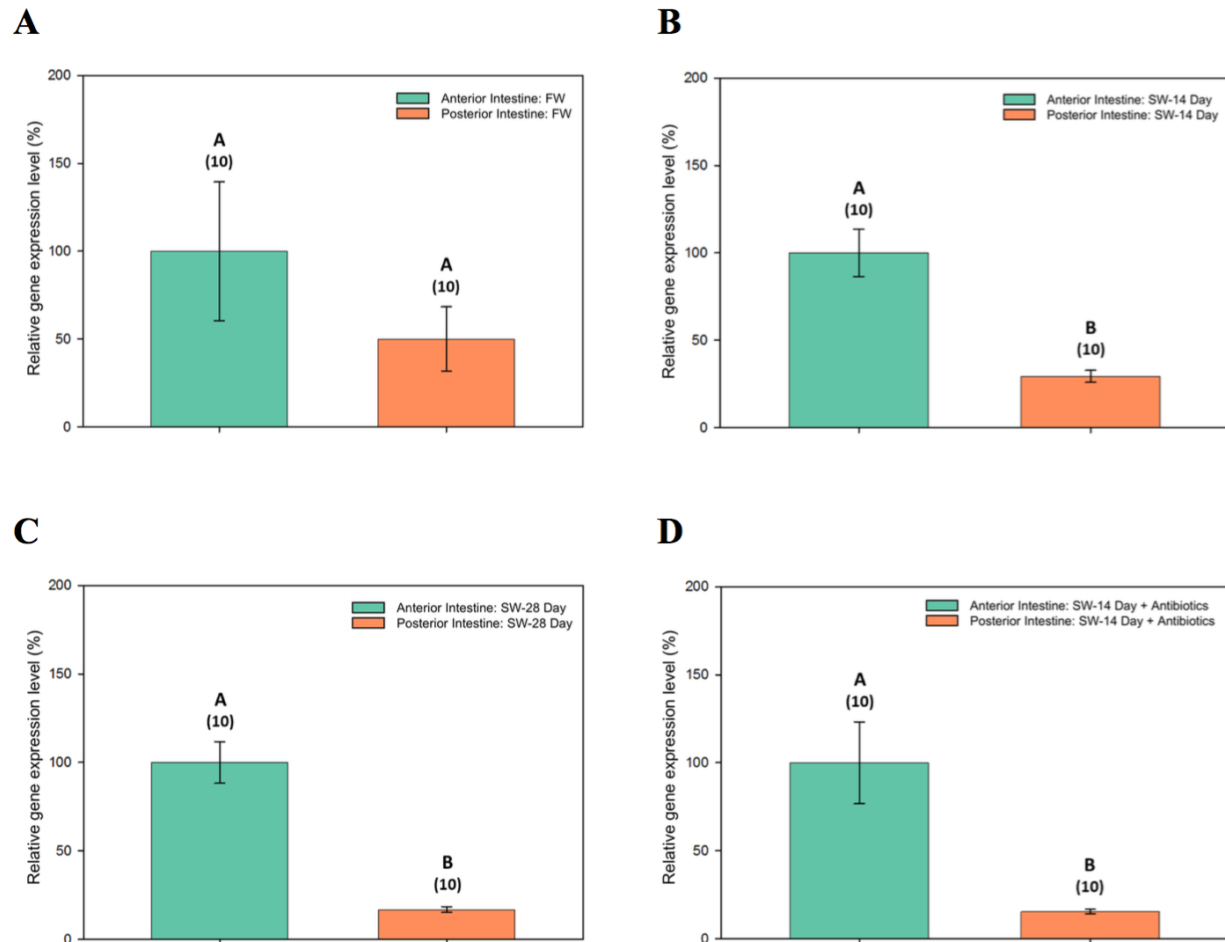


Figure 2.11 (A to D): Comparing relative SLC26A6 gene expression levels between the anterior and posterior intestines of the FW (A; %), SW-14 Day (B; %), SW-28 Day (C; %), and the SW-14 Day + Antibiotics (D; %) conditions. The relative gene expression levels are expressed as percentages with the anterior intestine in each condition set to 100%. The bars represent column means $\pm$ SEM and the numbers in the brackets represent n-values. Within each panel, bars that share letters are not statistically different. All data passed parametric parameters and the following statistical tests were used: Figure 2.11A: A paired, two-tailed t-test ($p=0.209$); Figure 2.11B: A paired, two-tailed t-test ($p<0.001$); Figure 2.11C: A paired, two-tailed t-test ($p<0.001$); Figure 2.11D: A paired, two-tailed t-test on $\log(10)$ -transformed data ($p<0.001$).

2.4 DISCUSSION

2.4.1 Summary

Acclimation to increased salinity impacted plasma and intestinal oxalate concentrations, while renal concentrations appeared unchanged. Further, elimination of bacteria from the intestine also resulted in plasma and intestinal oxalate concentration alterations, while oxalate concentrations in the kidney were once again unaffected. Finally, the mRNA expression of potential oxalate transporters, SLC26A6 and SLC26A3, was altered by salinity and antibiotic exposure in both the intestine and kidney.

2.4.2 Salinity Acclimation: Short-term (14-day) and long-term (28-day) salinity exposures

Acclimation to seawater had a significant, yet temporary, impact on oxalate homeostasis, first demonstrated by the initial increase in plasma oxalate concentrations following 14 days in seawater, followed by a return to control values after 28 days in seawater (Figure 2.3A); suggesting that oxalate regulation in fish is impacted by the environment but that physiologically, the animals are able to compensate given enough time. This is the first demonstration of plasma oxalate concentrations being altered in fish species, however plasma oxalate concentrations in mammals have been shown to be alterable through treatments such as dietary manipulation (e.g. Holmes *et al.*, 2005), through decreased renal health/capacity (e.g. Hatch *et al.*, 1994), and/or alteration of transporter expression through genetic manipulation (e.g. Neumeier *et al.*, 2020). For example, when human subjects consumed an oxalate supplemented diet there were time and dose dependent changes observed in the plasma oxalate concentration (Holmes *et al.*, 2005). Renal oxalate secretion plays a vital role in oxalate homeostasis, and plasma oxalate concentrations in humans

correlates directly with urinary oxalate excretion (Holmes *et al.*, 2005; Perinpam *et al.*, 2017; reviewed by Shah *et al.*, 2020).

Sailfin mollies appear to likewise use renal pathways to transport oxalate, as evidenced by detectable oxalate concentrations in the kidney of fish in both freshwater and seawater (Figure 2.3B). However, there were no corresponding correlation between the oxalate concentrations of the kidneys (Figure 2.3B) and altered plasma concentrations (Figure 2.3A) as would be predicted based on mammalian literature (Holmes *et al.*, 2005; Perinpam *et al.*, 2017; reviewed by Shah *et al.*, 2020). Interestingly, there was no detection of SLC26A3 expression in the kidneys of sailfin mollies acclimated to all four conditions (Figures 3.2A & 3.2B in the Appendix), suggesting that reabsorption of oxalate from renal filtrate is not likely, which is similar to studies on rats (Barmeyer *et al.*, 2007) and rabbits (Jacob *et al.*, 2002) as they also did not detect the presence of DRA (orthologue of mammalian SLC26A3), in the renal cortex of these animals (reviewed by Freel *et al.*, 2013). In contrast to sailfin mollies, rats, and rabbits, SLC26A3 was detected in the human kidney, but it is thought to play a reduced role compared to SLC26A3 in the intestine (Wedenoja *et al.*, 2008; reviewed by Freel *et al.*, 2013), corresponding to a reduced impact on renal oxalate handling in humans as well. Further, this finding is consistent with research on eels where SLC26A3 was expressed almost exclusively in the digestive tract and was absent in the kidney (Watanabe & Takei, 2011).

In contrast, SLC26A6 was highly expressed in the kidney and was increased through salinity acclimation (Figure 2.5A & 2.12B), as previously observed in obscure pufferfish (Kato *et al.*, 2009). However, there was no significant difference between the SW-14 Day and 28-day conditions which indicated that SLC26A6 expression was initially increased to meet needs in seawater and that long-term (28-day) salinity exposure did not affect this change (Figure 2.5A).

Further, this increase in expression was not accompanied by alterations in renal oxalate concentrations (Figure 2.3B) as would have been predicted based on the role of SLC26A6 in oxalate secretion (Freel *et al.*, 2006; Wang *et al.*, 2021). Indeed, this increase in secretion should have resulted in amelioration of plasma oxalate concentrations and an increase in renal oxalate concentrations, however neither of these results were evident in this study. This suggests that SLC26A6 may be acting on other transport products such as $\text{Cl}^-/\text{formate}$, Cl^-/OH^- , and $\text{Cl}^-/\text{HCO}_3^-$ (Knauf *et al.*, 2001; Jiang *et al.*, 2002; Thomson *et al.*, 2005; Xie *et al.*, 2002) and/or is not sufficient in oxalate transport capacity for a measurable impact. Additionally, as this study measured free oxalate concentrations, any increase in oxalate precipitation would not have been observed and cannot be ruled out. Regardless, these findings may indicate that the fish are utilizing their kidneys to handle oxalate but perturbations in plasma oxalate are ameliorated through additional pathways, such as the intestine.

In humans, the kidneys play the largest role in removing oxalate from the blood but some oxalate is also excreted through bile and the intestine (Brzica, *et al.*, 2013). Further, metabolic disorders such as primary hyperoxaluria and even the excessive ingestion of oxalate can result in a systemic oxalate burden, which in turn has been observed to trigger secretion of oxalate into the intestine in rats (Costello *et al.*, 1992; Hatch *et al.*, 1994, 2006, 2011; reviewed by Whittamore 2020). Specifically, six weeks after a 5/6 nephrectomy in rats, the distal colon functioned as the primary site for oxalate secretion, although it was also found that the whole intestinal tract could excrete oxalate in an adaptive response to compensate for the loss of efficient renal oxalate secretion (Hatch *et al.*, 1994). As renal oxalate concentrations did not change in response to salinity (Figure 2.3B) while salinity-dependent changes were observed in the intestinal oxalate concentrations (Figure 2.3C), this suggests that unlike in humans, the sailfin molly intestine may

be more involved in oxalate regulation during perturbations than the kidney. Indeed, evidence suggests that the piscine intestine may be able to transport far more oxalate than the mammalian intestine (Whittamore, 2020). Such high oxalate transport rates into the lumen of the seawater teleost intestine may result in oxalate being readily available to bind to calcium ions that are consumed from the surrounding seawater (Whittamore, 2020).

In contrast to the current study where salinity influenced the concentration of oxalate in the intestine, another study showed no impact of salinity on transport rates (Whittamore 2020). However, Whittamore's 2020 study used symmetrical, short-circuit conditions to observe epithelial oxalate transport across the sailfin molly intestine whereas the current *in vivo* study examined free oxalate concentrations in the intestine. Further, while we were unable to determine the concentration of oxalate in the intestinal fluid (and thus determine plasma-intestinal diffusion gradients), it is possible this study had asymmetrical conditions compared to Whittamore (2020). The symmetrical conditions of Whittamore's 2020 study would have been set-up to have the same plasma and intestinal oxalate concentrations and thus, no changes in diffusion rates were observed during salinity acclimation. However, the possible asymmetrical conditions in this current study could have resulted in greater diffusion of oxalate into the intestine during seawater acclimation. This could potentially explain the impact of salinity on the intestine in this study compared to Whittamore (2020).

Additionally, seawater teleosts continuously drink to replenish the water their bodies lose to their hyperosmotic environments while their intestine simultaneously absorbs water efficiently (Larsen *et al.*, 2014). However, the calcium from the imbibed seawater does not place a large osmoregulatory stress on the kidney as more than 80% of the calcium stays within the intestine and is subsequently excreted, while ~40-60% of this calcium is precipitated as calcium carbonate

(Shehadeh & Gorden, 1969; Wilson & Grosell 2003). Seawater teleosts possess this unique ability for enteric biomineralization (Wilson & Grosell, 2003) and recently in 2020, Whittamore proposed that this process may also be capturing oxalate. Thus, with increased salinity, there may be an increase in oxalate precipitation which would lower free oxalate concentrations. However, an increase in free oxalate was actually observed in the anterior intestine (Figures 2.3C & 2.3D), suggesting that the increase in oxalate in the intestine was potentially much greater than measured.

Since SLC26A3 is involved in net transcellular absorption in the mice intestine (Mukaibo, *et al.*, 2018), it was expected that expression will be highest in the FW condition and then decrease in the seawater conditions to aide in overall oxalate excretion. As predicted, for both the anterior and posterior intestines, SLC26A3 expression in both the seawater conditions (14-day and 28-day) was lower than the FW condition (Figures 2.4A, 2.4B, & 2.12B). Past and current literature regarding SLC26A3 expression in osmoregulatory organs, especially the intestine of seawater teleosts have varying results. SLC26A3 was previously found to be highly expressed in both the anterior and mid-intestine of tilapia acclimated to either freshwater or seawater however a clear pattern of intestinal SLC26A3 expression related to salinity was not evident (Ruiz-Jarabo *et al.*, 2017). Additionally, in the anterior intestine of sea bream, there were increases in SLC26A3 expression in a salinity dependent manner (i.e. SLC26A3 expression increased as salinity increased; Gregorio *et al.*, 2013). Further, while SLC26A3 expression was unaffected in the anterior intestine of red drum acclimated from freshwater to hypersalinity (60 g/kg; 4-hour and 72-hour exposure; Martin & Esbaugh, 2021), there was an increase in SLC26A3 expression in the posterior intestine of red drum 72-hours after transfer to the hypersaline condition (Martin & Esbaugh, 2021). Grosell and colleagues remarked in their review paper on HCO_3^- secretion and Cl^- absorption in the seawater teleost intestine that “the function of SLC26A3 and other anion

exchangers in the seawater fish intestinal tissue remains to be identified and clearly is an exciting avenue for future study” (2009). Perhaps as the sailfin molly is an herbivore compared to the red drum (carnivore; Gaylord & Gatlin, 1996) and sea bream (mainly carnivorous but also accessorially herbivorous; Kormas *et al.*, 2014), there is a corresponding impact on SLC26A3. As sailfin mollies may be consuming excess oxalate, the expression of this transporter may hence differ as increased dietary oxalate may require less oxalate absorption via SLC26A3, thus creating species-specific expression patterns.

During both short-term (14-day) and long-term (28-day) seawater acclimation, SLC26A6 expression increased in the anterior intestine; but there was again no significant difference in SLC26A6 expression between the two (14-day and 28-day) seawater conditions (Figures 2.5B & 2.12B) as seen with SLC26A3. In contrast, SLC26A6 expression showed a significant increase in the SW-14 Day condition (Figures 2.5C & 2.12B) in the posterior intestine; but SLC26A6 expression in the long-term seawater condition (SW-28 Day) was not significantly different from either the FW or SW-14 Day condition (Figure 2.5C). The results of Whittamore’s 2020 study support the findings in this study as seawater-acclimation led to higher SLC26A6 expression in both the anterior and posterior intestine as generally observed in this study. Seawater has high concentrations of divalent ions like Ca^{2+} ($\sim 16.10 \text{ mmol L}^{-1}$) and Mg^{2+} ($\sim 34.54 \text{ mmol L}^{-1}$) which enter the intestine as water is absorbed (Becker *et al.*, 2011). However, these divalent cations can accumulate in the intestine to toxic levels and in turn create an osmotic gradient that would negatively affect absorption of fluids (Wilson & Grosell, 2003). When HCO_3^- is secreted by the intestine, it can bind with Ca^{2+} and Mg^{2+} to form CaCO_3 and MgCO_3 , which are insoluble, no longer exert osmotic pressure and are eventually excreted from the intestine (Wilson *et al.*, 2002). Through this mechanism, water absorption in the intestine is facilitated (Wilson & Grosell, 2003),

which is important for seawater teleosts living in hyperosmotic environments. HCO_3^- secretion in the intestine has been shown to be stimulated when the amount of Ca^{2+} delivered to in the intestine is increased (Genz *et al.*, 2008). When salinity is increased, Ca^{2+} delivered to the intestine is also increased which in turn enhances intestinal bicarbonate secretion (Genz *et al.*, 2008). Since SLC26A6 is an anion exchanger involved in not only oxalate transport but also HCO_3^- secretion (Freel *et al.*, 2006; Mount & Romero, 2003), its expression was predicted and subsequently observed to increase in the both the anterior and posterior intestine of animals acclimated to seawater (Figures 2.5B, & 2.5C).

It is important to note that there are other potential oxalate transporters in the intestine that were not examined, such as SLC26A1 which has been observed to mediate oxalate secretion in the mice intestine (Dawson *et al.*, 2010). Thus, there could be other factors including anion exchangers and/or oxalate transporters (that are yet to be characterized and studied in the sailfin molly) that are involved in maintaining/mitigating rates of net oxalate flux across the sailfin molly intestine in both freshwater and seawater-acclimated animals.

Finally, as the oxalate concentrations of the anterior intestine and posterior intestine changed in response seawater acclimation, salinity clearly impacted the handling of oxalate in sailfin molly intestine. Interestingly, the gut microbiota of fish such as salmon has also been seen to change in response to salinity (e.g. Dehler *et al.*, 2017). For example, the abundance of *Lactobacillales* was 36.7% and 54.8% of all bacterial orders in the gut microbiome of freshwater and seawater-acclimated salmon respectively (Dehler *et al.*, 2017). In fact, *Lactobacillus* was found to be in dominant genera in the gut microbiome of seawater-acclimated salmon, representing 95.23% of the lactic acid bacteria but only 52.28% in freshwater-acclimated salmon (Dehler *et al.*, 2017). In contrast, *Lactobacillaceae* were in higher abundance in the intestines of freshwater-

acclimated salmon, (Zhao *et al.*, 2020). Since salinity has previously been shown to impact the abundance of *Lactobacillus* in fish (Dehler *et al.*, 2017; Zhao *et al.*, 2020), sailfin mollies from the SW-14 Day and SW-28 Day conditions may also have altered abundances of *Lactobacillus* (e.g. *L. acidophilus* and *L. casei*) that can degrade large amounts of oxalate (Hatch *et al.*, 2006; Miller & Dearing, 2013). If sailfin mollies from the seawater conditions (SW-14 Day and SW-28 Day) have an increased abundance of *Lactobacillus* than their counterparts in FW, efficient oxalate-degrading bacteria belonging to *Lactobacillus* could ameliorate the sailfin mollies' reduced ability for urinary oxalate excretion in seawater by degrading more oxalate in the intestine. Further, if this increase in bacteria abundance was located in the posterior intestine, it would explain the decreasing oxalate concentrations along the gut. The dominant bacteria in the gut microbiome of freshwater (0ppt and 5ppt) *Poecilia sphenops* (Mexican or black molly) was *Aeromonas* and *Cetobacterium* while *Vibrio* and *Enterobacteriaceae* were highest in abundance in the intestine those acclimated to saline (18ppt and 30ppt) conditions (Schmidt *et al.*, 2015) but none of these bacteria were tested for oxalate-degrading properties. Since the sailfin molly is very closely related to the black molly, it is highly likely that the sailfin mollies used in this study also experienced changes in their gut microbiomes when they were acclimated from freshwater to seawater conditions. Clearly, more work is needed to examine the intestinal bacteria more closely, including identifying the bacteria within the intestine and the impact of salinity.

2.4.3 Antibiotics Treatment: SW-14 Day + Antibiotics exposure

The removal of bacteria from the intestine had profound effects on oxalate handling in seawater sailfin mollies. The elevated plasma oxalate concentration in the SW-14 Day + Antibiotics condition correlated with the loss of oxalate-degrading bacteria in their gut (Figures 2.6A & 2.12C). Further, while the oxalate concentration in the anterior intestine of the SW-14

Day + Antibiotics condition was significantly lower than the SW-14 Day condition suggesting that the removal of oxalate degrading bacteria lowered free oxalate concentrations (which was not predicted), antibiotic exposure increased the oxalate concentrations in the posterior intestine as hypothesized (Figures 2.6C & 2.6D). Within the human gut microbiome, *O. formigenes* is one of the main bacteria involved in oxalate degradation as well as *Bifidobacterium*, *Lactobacillus*, and *Escherichia coli* (Kaufman *et al.*, 2008). Kaufman and colleagues' 2008 study found that when the human intestine was colonized with *O. formigenes*, there was an associated 70% decrease in the risk of becoming a recurrent kidney/calcium oxalate stone former. An issue that has arisen with the overuse of oral antibiotics is that *O. formigenes*, a bacterium capable of degrading large amounts of oxalate in the human gut, is very susceptible to commonly prescribed antibiotics (e.g. doxycycline, gentamicin, tetracycline, clindamycin; Lange *et al.*, 2012). Even though the gut contents of the sailfin molly previously tested negative for *O. formigenes*, there are likely still oxalate-degrading bacteria found within the intestinal microbiota of these fish (Allison *et al.*, 1985; reviewed by Whittamore, 2020). However, the main bacterium or bacteria involved in degrading the highest amounts of oxalate within the sailfin molly gut microbiome is still unknown and would be an interesting avenue for future studies. Further, while past literature has not reported on the presence of *O. formigenes* in the gut microbiome of fish, one study found that *O. formigenes* was present in the gills of Asian sea bass (the intestine was not checked for the presence/absence of this bacterium; Gomes *et al.*, 2019). Interestingly, there was a higher abundance of *O. formigenes* in the gills of Asian sea bass that had high infection levels of *Chilodonella hexasticha* (as observed by cell counts of *C. hexasticha* in the gills) than fish that were not infected by this parasite (Gomes *et al.*, 2019), which may mean that *O. formigenes* may be more of a pathogenic bacterium in fish.

This was the first study to use antibiotic treatments on fish to observe the effects it has on oxalate degradation, transport, and excretion. However, human research suggests that increased antibiotic consumption is closely correlated with increased kidney stone formation (Ferraro *et al.*, 2019; Tasian *et al.*, 2018; Zampini *et al.*, 2019) which is likely driven by excess oxalate (Nazzari & Blaser, 2018). These findings regarding antibiotic-use in mammals suggests that sailfin mollies could also be facing an increased risk of kidney stone formation in the SW-14 Day + Antibiotics condition. However, the oxalate concentration of the kidney in the SW-14 Day + Antibiotics condition was not significantly different from the SW-14 Day condition (Figure 2.6B) once again despite elevated plasma levels. The enhanced excretion of oxalate could have resulted in increased oxalate precipitates as in mammals (Freel *et al.*, 2006) which would not have been detectable using the current methods (only free oxalate is measured). Perhaps the elevated level of SLC26A6 expression in the kidneys of SW-14 Day + Antibiotics fish compared to their counterparts in the SW-14 Day condition could be due to the animals' compensating for the loss of oxalate-degrading bacteria in the intestine (Figures 2.8A & 2.12C) and thus, enhanced renal secretion of oxalate via SLC26A6 may be necessary to restore homeostasis in the plasma and this secretion is captured as precipitates.

Since SLC26A3 is involved in oxalate absorption (Freel *et al.*, 2013), it was predicted that there would be a further downregulation of SLC26A3 in the SW-14 Day + Antibiotics treatment compared to the SW-14 Day condition which is what was observed (Figures 2.7A, 2.7B, & 2.12C). Overall this suggests that less oxalate is being absorbed by the intestine and can be seen by the increased oxalate concentration in the posterior intestine (Figure 2.6D), but in the anterior intestine, the oxalate concentration decreased in response to antibiotic treatment (Figure 2.6C). The possible explanation for this is unknown at present, but perhaps there is increased precipitation

of oxalate in the intestine due to osmoregulatory needs as discussed previously. Without oxalate degrading bacteria perhaps oxalate precipitation was enhanced and more work is needed to confirm this hypothesis.

Since SLC26A6 is involved in oxalate secretion in the intestine (Freel *et al.*, 2006), it was expected that there would be a further upregulation of SLC26A6 in the SW-14 Day + Antibiotics treatment compared to the SW-14 Day treatment to compensate for the loss of oxalate-degrading bacteria in the gut. However, the opposite was observed in both anterior and posterior sections of the intestine which could mean that the antibiotics were directly affecting SLC26A6 expression in an unanticipated way (Figures 2.8B, 2.8C, & 2.12C). Perhaps, if the antibiotics increased the pH of the intestine, the need for HCO_3^- secretion would be reduced and intestinal water absorption would occur as a part of the osmoregulation strategies employed by the teleost. This in turn would result in downregulation of SLC26A6 as less HCO_3^- is required. However, the pH of the antibiotic solution did not impact the pH of the seawater (measured with pH meter both before addition of antibiotics and after) and would not be likely to alter the pH of the intestine. Also, the pH of the 100mL antibiotic-seawater solution (pH=6.92) was lower than the pH of the seawater teleost intestine (pH ~8-9; Kurita *et al.*, 2008). Therefore, if the antibiotic treatment had any effect on the pH of the intestine, it would lower the pH of the intestine which would result in less precipitation of oxalate. Further, In 2004, Nozawa and colleagues studied the role of mouse PAT1 (putative anion transporter 1, orthologue of human SLC26A6) in the absorption of organic ions in the intestine. An interesting observation was that the uptake of formate by PAT1 was not affected by pH (Nozawa *et al.*, 2004). Further, they also demonstrated that pH does not affect the functionality of PAT1 (Nozawa *et al.*, 2004). However, the model organism of Nozawa and colleagues' 2004

study was the mouse and thus, there still needs to be more research on the effect of antibiotic treatments on not only the pH of the luminal intestine but also SLC26A6 expression in teleosts.

2.4.4 Zonation: Comparing the anterior and posterior intestines across all conditions

There was clear zonation of oxalate concentrations along the intestine of sailfin mollies. In freshwater and after exposure to antibiotics in seawater, the concentration increased along the intestine, while in seawater the opposite was observed (Figure 2.9). In a study of the gastrointestinal tract of the white-throated woodrat (*Neotoma albigula*), it was observed that the highest density of potential oxalate-degrading bacteria was found in the foregut (Miller *et al.*, 2014). This study also found that the combined oxalate-degrading genera of bacteria decreased throughout the length of digestive tract (Miller *et al.*, 2014). The higher density of oxalate degrading bacteria seen in the foreguts of rats (Miller *et al.*, 2014) could be similar in FW sailfin mollies and thus, the intestinal oxalate concentration of the anterior intestine was observed to be lower than the posterior (Figure 2.9A). Also, the abundance of oxalate-degrading bacteria was shown to drop off as you moved through the gastrointestinal tract of rats (Miller *et al.*, 2014). If this finding in rats was applied to FW (Figure 2.9A) sailfin mollies, it could help explain why the posterior intestine had a higher oxalate concentration than the anterior intestine as there could have been reduced amounts of oxalate-degrading bacteria in the posterior intestine compared to the anterior intestine. Even though there may be loss of bacteria in the intestine of SW-14 Day + Antibiotics sailfin mollies, there could still be comparatively more bacteria, including oxalate degrading bacteria in the anterior intestine than the posterior, which might have resulted in a higher oxalate concentration in the posterior intestine than the anterior intestine (Figure 2.9D).

Approximately 4-6 hours after ingestion of foods containing oxalate, humans experience a peak in oxalate absorption and the specific timing of this initial peak suggests that most oxalate

absorption occurs within the intestine, particularly the small intestine (Prenen *et al.*, 1984; Barilla *et al.*, 1978; Lindsjo *et al.*, 1989; reviewed by Grentz & Massey, 2002). Thus, it was predicted that the anterior intestine of sailfin mollies may be primarily responsible for oxalate handling in sailfin mollies. In agreement with the prediction, the oxalate concentrations of the anterior intestine of SW-14 Day (Figure 2.9B) and SW-28 Day (Figure 2.9C) sailfin mollies were higher than the posterior intestine of their respective condition, and this may indicate that the anterior intestine of these fish is also tasked with handling large amounts of oxalate (Brzica, *et al.*, 2013; Williams & Wilson, 1990), when the ability for urinary oxalate excretion is reduced in seawater conditions. Further, in all four conditions (FW, SW-14 Day, SW-28 Day, and SW-14 Day + Antibiotics), the anterior intestine had significantly higher SLC26A3 expression levels than the posterior intestine (Figure 2.10), as previously observed in the sea bream (Gergorio *et al.*, 2013). In contrast, SLC26A6 appears to exhibit a less consistent expression pattern along the intestine at least in FW as there was no statistically significant difference in SLC26A6 expression between the anterior and posterior intestine (Figure 2.11A). However, in the remaining three seawater conditions (SW-14 Day, SW-28 Day, and SW-14 Day + Antibiotics), the anterior intestine had significantly higher SLC26A6 expression levels than the posterior intestine (Figures 2.11B, 2.11C & 2.11D) as was seen with SLC26A3. SLC26A6 expression being consistently higher in the anterior intestine than the posterior intestine of sailfin mollies across all three seawater conditions (SW-14 Day, SW-28 Day, and SW-14 Day + Antibiotics; Figure 2.10) is also in agreement with findings of Gregorio and colleagues work on seawater sea bream (2013).

The observed expression pattern of both SLC26A3 (decreasing) and SLC26A6 (stable) in freshwater would combine to create an increasing concentration of oxalate along the intestine, which is exactly what is observed. However, the transporter expression pattern in seawater is not

as clear with the anterior intestine showing the highest levels of both reabsorption transporters and secretory transporters, compared to the posterior intestine. As the concentration decreases along the intestine in seawater there are a few possible explanations that require future work. First, the pH along the intestine of sailfin mollies is not known. If the pH was lower in the anterior intestine, more free oxalate would be present. This is unlikely however as SLC26A6 is also involved in transporting HCO_3^- ions out of the cell (Figure 2.2; Wang *et al.*, 2021; Sayer, 2017) and thus the pH of the intestine would likely be higher in the anterior portion thus resulting in less free oxalate. In fact, if this prediction is true, the measured amounts of oxalate handled by the anterior intestine are likely an underestimate. Indeed, in the intestine of seawater fish, anion exchange activity is thought to be “most pronounced” in the anterior intestine (Taylor & Grosell, 2006; Marshall & Grosell, 2006; Grosell & Genz, 2006) and even though all parts of the intestine in seawater fish have been observed to have capacity to exchange anions, the anterior intestine is the section that “predominantly” secretes HCO_3^- and absorbs Cl^- ions through anion exchange (Grosell, 2006). Secondly, the relative oxalate transport rates of SLC26A3 vs SLC26A6 are not known in fish. Perhaps SLC26A3 plays a lesser overall role. If that were to hold true, then the decreasing expression pattern of SLC26A6 would explain the decreasing concentration along the gut.

Whittamore also conducted a pilot study on European sea bass and turbot (*Scophthalmus maximus*) in which excreted calcium precipitates were collected, dissolved in HCl, and the subsequent liberated oxalate was determined using enzymatic assays (Trinity Biotech Ireland, Whittamore, 2020). For the European sea bass and turbot, the oxalate excretion was $4.6 \text{ nmol kg}^{-1} \text{ h}^{-1}$ and $5.5 \text{ nmol kg}^{-1} \text{ h}^{-1}$ respectively (Whittamore, 2020). However, this study was conducted on starved European sea bass and turbot, which suggests that the collected oxalate is mainly from endogenous sources and is secreted across these fish’s intestine (Whittamore, 2020). Whittamore’s

pilot study on European sea bass and turbot (2020) greatly differs from this study as sailfin mollies were fed daily while the European sea bass and turbot were starved. Also, Whittamore collected precipitated CaCO_3 which was subsequently dissolved in HCl while in this study, the concentration of “free” or unprecipitated oxalate was measured in the intestines of sailfin mollies. It would be interesting for future studies to compare the differences in oxalate concentrations and oxalate excretion rates ($\text{nmol kg}^{-1} \text{ h}^{-1}$) of both starved and fed fish as a way to observe the effects of endogenous and exogenous sources of oxalate on the fish intestine. Further, the oxalate concentrations of the diet and the subsequent impact on physiology should be explored. Perhaps the species-specific transporter expression patterns are related to species-specific dietary oxalate concentrations.

2.4.5. Conclusions

Overall, salinity appeared to have significant impacts on oxalate homeostasis, in particular affecting the plasma and intestinal concentrations by temporarily increasing circulating oxalate and reversing oxalate gradients along the intestine. Further, antibiotics also showed significant impacts on oxalate homeostasis, likewise increasing plasma oxalate concentrations and reversing zonation along the intestine to those similar to those observed in freshwater. Transporter expression was impacted in the intestine and kidney in response to both salinity and antibiotics. While some hypotheses were supported, such as increased excretion to the intestine in seawater, and decreased absorption through SLC26A3 expression, others were contradicted by the results. Further work is needed to examine total oxalate concentrations and identify specific abundances of oxalate degrading bacteria in the intestine.

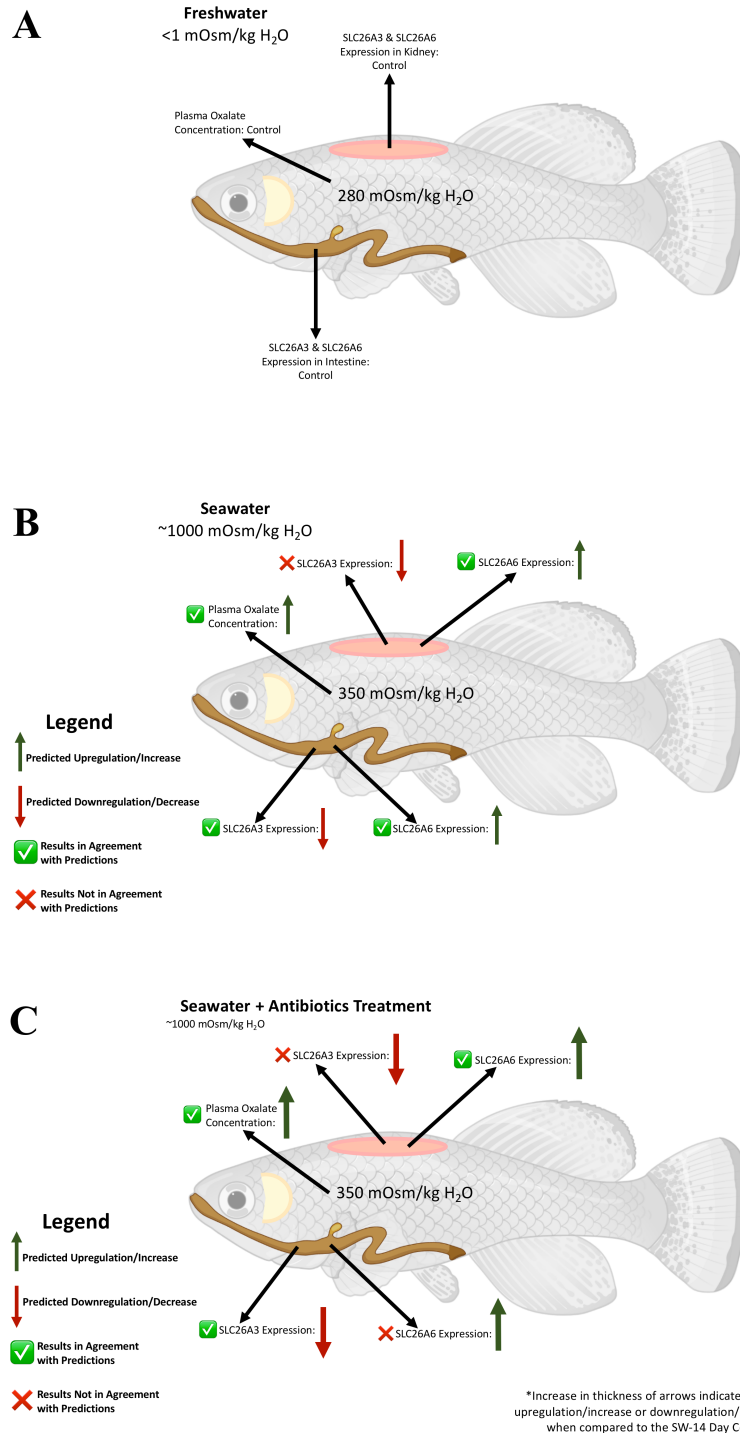


Figure 2.12 (A to C): A diagram illustrating the overall findings of this study and whether the hypotheses were supported or refuted by the results for the freshwater (A), seawater (B), and seawater-antibiotic treatment conditions (C). Predictions regarding the plasma oxalate concentrations (μM), SLC26A3 expression, and SLC26A6 expression for the seawater acclimation and seawater-antibiotic treatment condition are shown. These illustrations were created using biorender.com.

CHAPTER 3: CONCLUDING REMARKS AND FUTURE DIRECTIONS

3.1 Concluding remarks and significance of project

This was the first study to directly measure oxalate concentrations of the anterior intestines, posterior intestines, kidneys, and plasma of a euryhaline teleost. SLC26A3 and SLC26A6 are two important anion exchangers that involved in oxalate transport and the effect(s) of salinity and antibiotic treatment on the expression levels of SLC26A3 and SLC26A6 were observed for the first time. Majority of studies on fish also focus on those that are carnivorous or omnivorous. However, sailfin mollies are herbivorous fish (Courtenay & Meffe, 1989) that could potentially be consuming high levels of oxalate through their plant-based diet. This was also the first study to use broad-spectrum antibiotics to indirectly examine the role of the gut microbiome, specifically oxalate-degrading bacteria in oxalate degradation and excretion in the sailfin molly.

3.2 Limitations of the study

3.2.1 Attempting urine collection

Measuring the oxalate concentrations of urine from animals acclimated to each of the four conditions (FW, SW-14 Day, SW-28 Day, and SW-14 Day + Antibiotics) would have enabled us to have directly observe the effect of salinity and/or antibiotic treatment on urinary oxalate excretion as opposed to kidney concentrations. Pipette tips and western blot tips were melted and fused onto syringes in an attempt to collect urine from the urogenital pore. Glass capillary tubes were also used but they did not effectively collect urine from the urogenital pore either. None of these attempts yielded enough urine for use in the *Oxalate Assay Kit*. This limitation of being unable to collect urine could be due to sailfin mollies' small body size and their subsequent production of relatively small volumes of urine. The urogenital pore of the sailfin molly is very

small and if a capillary tube or pipette tube was designed to fit into this pore, it may be possible to collect urine, pool the urine per treatment condition and then the urinary oxalate concentrations could be measured. It would be very interesting to observe the changes in oxalate concentration in the urine of sailfin mollies acclimated to each of the four conditions (FW, SW-14 Day, SW-28 Day, and SW-14 Day + Antibiotics). Measuring oxalate levels in the urine will help us increase our knowledge of urinary oxalate excretion and if/how short-term and long-term salinity, as well as antibiotic treatments affect urinary oxalate concentrations. Urine collection should be attempted on larger euryhaline teleosts (*Chanos chanos*, *Oreochromis mossambicus*, etc...) as it will most likely yield larger volumes of urine which can be pooled. Then, the oxalate concentrations of the urine can be measured and compared.

3.2.2 *SLC26A3 and SLC26A6 have many substrates*

The SLC26 gene family codes for anion exchangers that are involved in the transport of Cl^- , I^- , SO_4^{2-} , OH^- , HCO_3^- , formate, and oxalate (Mount & Romero, 2003). SLC26A6 and SLC26A3 code for apical anion exchangers for which oxalate has been observed to be a substrate (Kurita *et al.*, 2008). However, the extent to which SLC26A3 and SLC26A6 transport oxalate versus other substrates has not been studied in teleosts. Therefore, any changes in SLC26A3 and SLC26A6 expression in response to salinity and/or antibiotic treatment cannot solely be attributed to facilitating oxalate transport as these anion exchangers are involved in transporting other substrates as well. More research needs to be done on SLC26A3 and SLC26A6 to study the mechanism(s) by which these proteins transport not only oxalate but also their other substrates.

3.2.3 Free oxalate vs. total oxalate

In this study, free oxalate concentrations were measured. This did not reveal any potential changes in precipitated oxalate concentrations. Alterations in renal and/or intestinal handling of oxalate thus potentially underestimate total oxalate handling. Increasing in oxalate excretion captured as precipitates is hence unknown and an important aspect of understating the impacts of salinity and antibiotics on total oxalate handling.

3.3 Future directions

There has been very little research on oxalate handling and transport in euryhaline teleosts. It would be very interesting to study the effect of salinity, antibiotic-treatment, and even an oxalate-supplemented diet on oxalate concentrations in the urine as this would enable us to directly measure urinary oxalate excretion. Sailfin mollies are herbivorous teleosts (Courtenay & Meffe, 1989) and thus are potentially consuming more oxalate via their diet than their omnivorous and carnivorous counterparts. Studying the impact of an oxalate-supplemented diet on oxalate levels in the intestines, kidneys, and plasma would allow us to compare the effect of increasing exogenous sources of oxalate versus a normal balance of endogenous and exogenous oxalate sources.

Since antibiotic treatment would lower the number of oxalate-degrading bacteria in the gut, SLC26A6 expression was predicted to be higher in the intestines of sailfin mollies in the SW-14 Day + Antibiotics treatment than those acclimated to the SW-14 Day condition (Figures 2.1E & 2.1F). In contrast to these predictions, intestinal SLC26A6 expression was found to be lower in animals from SW-14 Day + Antibiotics condition than those acclimated to the SW-14 Day condition (Figure 2.12C). This finding needs to be further researched as there are no past studies that examined the direct effect of antibiotic treatments on intestinal SLC26A6 expression in

teleosts (or mammals). Since past studies have linked the consumption of antibiotic treatments to increased risk of kidney stones in humans (Ferraro *et al.*, 2019), it would also be interesting to observe the expression levels of SLC26A3 and SLC26A6 in their intestines and kidneys.

SLC26A3 expression was not detected in the kidneys of sailfin mollies. Similarly, Watanabe and Takei observed that in euryhaline eels, SLC26A3 was almost exclusively expressed within the digestive tract and expression was absent within the kidneys (2011). Future research should focus on determining if SLC26A3 expression is absent in the kidneys of many other euryhaline teleosts and whether it is a commonality amongst these group of fishes. Future research should also focus on elucidating the effects of antibiotic treatments on SLC26A3 and SLC26A6 expression in the intestines and kidneys of fish, especially euryhaline teleosts so that patterns in gene expression can be compared across species.

There are bacteria that have been characterized to degrade large quantities of oxalate such as *O. formigenes*, *L. acidophilus*, and *L. casei* (Hatch *et al.*, 2006; Miller & Dearing, 2013). In mammals, *O. formigenes* is thought to be one of the main oxalate-degrading bacteria in the gut (Hatch *et al.*, 2006, 2011). The gut contents of sailfin mollies have tested negative for *O. formigenes* (Allison *et al.*, 1985). Future research should focus on identifying which oxalate-degrading bacteria are the most efficient at degrading oxalate within the sailfin molly gut microbiome. Since the teleost intestine has been proposed to be a major excretory pathway for oxalate (Whittamore, 2020), there should also be studies comparing the types and abundance of oxalate-degrading bacteria within the gut microbiota of euryhaline teleosts. Using euryhaline teleosts as model organisms will enable us to observe the effect(s) of salinity on the expression of oxalate transporters as well as any changes in the abundance/localization of oxalate-degrading bacteria in the intestine.

Salinity has also been shown to have major effects on the gut microbiota of fish. For example, the transfer of Atlantic salmon (Dehler *et al.*, 2017), Nile tilapia (Zhang *et al.*, 2016), and Mexican mollies (Schmidt *et al.*, 2015) from freshwater to seawater resulted in the intestinal microbiota profiles of these fish being altered. Therefore, it would also be very interesting to study if the localization and abundance of oxalate-degrading bacteria in the euryhaline teleost's intestine changes in response to seawater acclimation.

Finally, future studies must be conducted to observe the total oxalate concentrations to reveal the impact of salinity and/or antibiotics on precipitated oxalate. Due to the role of precipitated oxalate in osmoregulation and kidney stone formation, this information will reveal important facets that were obscured in the current study.

REFERENCES

- Allison MJ, Dawson KA, Mayberry WR, Foss JG. 1985. *Oxalobacter formigenes*: oxalate-degrading anaerobes that inhabit the gastrointestinal tract. *Archives of Microbiology*. 141(1): 1-7. doi: 10.1007/bf00446731.
- Alper SL & Sharma AK. 2013. The SLC26 gene family of anion transporters and channels. *Molecular Aspects of Medicine*. 34(2-3): 494-515. doi: <https://doi.org/10.1016/j.mam.2012.07.009>.
- Alves A, Gregório SF, Egger RC, Fuentes J. 2019. Molecular and functional regionalization of bicarbonate secretion cascade in the intestine of the European sea bass (*Dicentrarchus labrax*). *Comparative Biochemistry and Physiology – Part A: Molecular & Integrative Physiology*. 233: 53-64. doi: <https://doi.org/10.1016/j.cbpa.2019.03.017>.
- Ando M, Wong MKS, Takei Y. 2014. Mechanisms of guanylin action on water and ion absorption at different regions of seawater eel intestine. *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology*. 307(6): R653-R663. doi: <https://doi.org/10.1152/ajpregu.00543.2013>.
- Asplin JR. 2002. Hyperoxaluric calcium nephrolithiasis. *Endocrinology and Metabolism Clinics of North America*. 31(4): 927-949. doi: 10.1016/s0889-8529(02)00030-0.
- Barilla DE, Notz C, Kennedy D, Pak CY. 1978. Renal oxalate excretion following oral oxalate loads in patients with ileal disease and with renal and absorptive hypercalciurias. *American Journal of Medicine*. 64(4): 579-585. doi: 10.1016/0002-9343(78)90576-4.
- Barmeyer C, Ye JH, Sidani S, Geibel J, Binder HJ, Rajendran VM. 2007. Characteristics of rat downregulated in adenoma (rDRA) expressed in HEK 293 cells. *Pflugers Archives*. 454(3): 441-450. doi: 10.1007/s00424-007-0213-7.
- Bayaa M, Vulesevic B, Esbaugh A, Braun M, Ekker ME, Grosell M, Perry SF. 2009. The involvement of SLC26 anion transporters in chloride uptake in zebrafish (*Danio rerio*) larvae. *Journal of Experimental Biology*. 212(20): 3283-3295. doi: <https://doi.org/10.1242/jeb.033910>.
- Becker AG, Goncalves JF, Toledo JA, Burns MD, Garcia LO, Vieira JP, Baldisserotto B. 2011. Plasma ion levels of freshwater and marine/estuarine teleosts from Southern Brazil. *Journal of Neotropical Ichthyology*. 9(4): 895-900. doi: <https://doi.org/10.1590/S1679-62252011005000039>.
- Beyenbach KW. 1995. Secretory electrolyte transport in renal proximal tubules of fish. In *Cellular and Molecular Approaches to Fish Ionic Regulation* (ed. Wood CM, Shuttleworth TJ). pp. 85-105. San Diego: Academic Press.

- Beyenbach KW. 2004. Kidneys sans glomeruli. *American Journal of Physiology-Renal Physiology*. 286(5): 811-827. doi: 10.1152/ajprenal.00351.2003.
- Blazer VS & Wolke RE. 1983. Ceroid deposition, retinal degradation and renal calcium oxalate crystals in cultured clownfish, *Amphiprion ocellaris*. *Journal of Fish Diseases*. 6(4): 365-376. doi: <https://doi.org/10.1111/j.1365-2761.1983.tb00088.x>.
- Brown PB, Kaushik SJ, Peres H. 2008. Protein feedstuffs originating from soybeans. In *Alternative Protein Sources in Aquaculture Diets* (ed. Lim C, Webster CD and Lee C). pp. 205-223. New York: Haworth Press.
- Brzica H, Breljak D, Burckhardt BC, Burckhardt G, Saboli I. 2013. Oxalate: from the environment to kidney stones. *Archives of Industrial Hygiene and Toxicology*. 64(4): 609-630. doi: <https://doi.org/10.2478/10004-1254-64-2013-2428>.
- Butt RL & Volkoff H. 2019. Gut microbiota and energy homeostasis in fish. *Frontiers in Endocrinology*. 10(9). doi: 10.3389/fendo.2019.00009.
- Caruso G. 2015. Use of plant products as candidate fish meal substitutes: an emerging issue in aquaculture productions. *Fisheries and Aquaculture Journal*. 6(3): e123. doi: <http://dx.doi.org/10.4172/2150-3508.1000e123>.
- Chourasia TK, D'Cotta H, Baroiller J, Slosman T, Cnaani A. 2018. Effects of the acclimation to high salinity on intestinal ion and peptide transporters in two tilapia species that differ in their salinity tolerance. *Journal of Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology*. 218: 16-23. doi: 10.1016/j.cbpa.2018.01.004.
- Costello JF, Smith M, Stolarski C, Sadovnic MJ. 1992. Extrarenal clearance of oxalate increases with progression of renal failure in the rat. *Journal of the American Society of Nephrology*. 3(5): 1098-1104. doi: <https://doi.org/10.1681/ASN.V351098>.
- Courtenay WR & Meffe GK. 1989. Small fishes in strange places: a review of introduced poeciliids. In *Ecology and Evolution of Livebearing Fishes, Poeciliidae* (ed. Courtenay WR & Meffe GK). pp. 319–331. Englewood Cliffs: Prentice Hall.
- Dawson PA, Russell CS, Lee S, McLeay SC, Van-Dongen JM, Cowley DM, Clarke LA, Markovich D. 2010. Urolithiasis and hepatotoxicity are linked to the aCnion transporter SAT1 in mice. *Journal of Clinical Investigation*. 120(3): 706-712. doi: <https://doi.org/10.1172/JCI31474>.
- Dehler CE, Secombes CJ, Martin SAM. 2017. Seawater transfer alters the intestinal microbiota profiles of Atlantic salmon (*Salmo salar* L.). *Scientific Reports*. 7: 13877. doi: <https://doi.org/10.1038/s41598-017-13249-8>.
- Dyer MH. “Feeding fish plants - gardening know how.” *Gardening Know How*. 24 Aug. 2020. <https://www.gardeningknowhow.com/ornamental/water-plants/wgen/feeding-fish-plants.htm>.

- Evans DH, Piermarini PM, Choe KP. 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiological Reviews*. 85(1): 97-177. doi: <https://doi.org/10.1152/physrev.00050.2003>.
- Edwards SL & Marshall WS. 2013. Principles and patterns of osmoregulation and euryhalinity in fishes. In *Fish Physiology: Euryhaline Fishes*. Volume 32 (ed. McCormick SD, Farrell AP, Brauner CJ). pp. 1-44. Massachusetts: Academic Press.
- Esbaugh AJ & Cutler B. 2016. Intestinal Na^+ , K^+ , 2Cl^- cotransporter 2 plays a crucial role in hyperosmotic transitions of a euryhaline teleost. *Physiological Reports*. 4(22): e13028. doi: <https://doi.org/10.14814/phy2.13028>.
- Fargue S, Knight J, Holmes RP, Rumsby G, Danpure CJ. 2016. Effects of alanine: glyoxylate aminotransferase variants and pyridoxine sensitivity on oxalate metabolism in a cell-based cytotoxicity assay. *Biochimica Et Biophysica Acta (BBA) - Molecular Basis of Disease*. 1862(6): 1055-1062. doi:10.1016/j.bbadis.2016.02.004.
- Ferraro PM, Curhan GC, Gambaro G, Taylor EN. 2019. Antibiotic use and risk of incident kidney stones in female nurses. *American Journal of Kidney Diseases*. 74(6): 736-741. doi: 10.1053/j.ajkd.2019.06.005.
- Ferreira-Martins D, Wilson JM, Kelly SP, Kolosov D, McCormick SD. 2021. A review of osmoregulation in lamprey. *Journal of Great Lakes Research*. 47(1): S59-S71. doi: <https://doi.org/10.1016/j.jglr.2021.05.003>.
- Food and Agriculture Organization (FAO). “Part 1: World review.” *The state of world fisheries and aquaculture*. United Nations. 2020. https://www.fao.org/3/ca9229en/online/ca9229en.html#chapter-1_1.
- Freel RW, Hatch M, Green M, Soleimani M. 2006. Ileal oxalate absorption and urinary oxalate excretion are enhanced in SLC26A6 null mice. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 290(4): 719-728. doi:10.1152/ajpgi.00481.2005.
- Freel RW, Morozumi M, Hatch M. 2009. Parsing apical oxalate exchange in Caco-2BBel1 monolayers: SiRNA knockdown of SLC26A6 reveals the role and properties of PAT-1. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 297(5): 918-929. doi:10.1152/ajpgi.00251.2009.
- Freel RW, Whittamore J, Hatch M. 2013. Transcellular oxalate and Cl^- absorption in mouse intestine is mediated by the DRA anion exchanger SLC26A3 and DRA deletion decreases urinary oxalate. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 305(7): 520-527. doi:10.1152/ajpgi.00167.2013.
- Gallo BD, Farrell JM, Leydet BF. 2020. Fish gut microbiome: a primer to an emerging discipline in the fisheries sciences. *Fisheries*. 45(5): 271-282. doi: <https://doi.org/10.1002/fsh.10379>.

- Gallo BD, Farrell JM, Leydet B. 2020. Use of next generation sequencing to compare simple habitat and species level differences in the gut microbiota of an invasive and native freshwater fish species. *PeerJ: Life & Environment*. 8: e10237. doi: 10.7717/peerj.10237.
- Ganong WF. 1989. Regulation of gastrointestinal function. In *Review of Medical Physiology* (Ed. 14). pp. 408-435. London: Prentice Hall.
- Garcia-Perez I, Villasenor A, Wijeyesekera A, Posma JM, Jiang Z, Stamler J, Aronson P, Unwin R, Barbas C, Elliott P, Nicholson J, Holmes E. 2012. Urinary metabolic phenotyping the SLC26A6 (chloride–oxalate exchanger) null mouse model. *Journal of Proteome Research*. 11(9): 4425-4435. doi: <https://doi.org/10.1021/pr2012544>.
- Gaylord TG & Gatlin DM. 1996. Determination of digestibility coefficients of various feedstuffs for red drum (*Sciaenops ocellatus*). *Aquaculture*. 139(3-4): 303-314. doi: [https://doi.org/10.1016/0044-8486\(95\)01175-7](https://doi.org/10.1016/0044-8486(95)01175-7).
- Genz J, Esbaugh, Grosell M. 2011. Intestinal transport following transfer to increased salinity in an anadromous fish (*Oncorhynchus mykiss*). *Journal of Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology*. 159(2): 150-158. doi: <https://doi.org/10.1016/j.cbpa.2011.02.011>.
- Genz J, Taylor JR, Grosell M. 2008. Effects of salinity on intestinal bicarbonate secretion and compensatory regulation of acid–base balance in *Opsanus beta*. *Journal of Experimental Biology*. 211(14): 2327-2335. doi: <https://doi.org/10.1242/jeb.016832>.
- Gilbert K. “List of euryhaline organisms.” *WorldAtlas-Environment*. 6 June 2017. www.worldatlas.com/articles/list-of-euryhaline-organisms.html.
- Gomes GB, Hutson KS, Domingos JA, Villamil SI, Huerlimann R, Miller TL, Jerry DR. 2019. Parasitic protozoan interactions with bacterial microbiome in a tropical fish farm. *Aquaculture*. 502: 196-201. doi: <https://doi.org/10.1016/j.aquaculture.2018.12.037>.
- Gonzalez RJ, Cooper J, Head D. 2005. Physiological responses to hyper-saline waters in sailfin mollies (*Poecilia latipinna*). *Journal of Comparative Biochemistry and Physiology – Part A: Molecular and Integrative Physiology*. 142(4): 397-403. doi: <https://doi.org/10.1016/j.cbpa.2005.08.008>.
- Greenwell MG, Sherrill J, Clayton LA. 2003. Osmoregulation in fish. *veterinary clinics of North America: exotic animal practice*. 6(1): 169-189. doi:10.1016/s1094-9194(02)00021-x.
- Gregorio SF, Carvalho ESM, Encarnacao S, Wilson JM, Power DM, Canario AVM, Fuentes J. 2013. Adaptation to different salinities exposes functional specialization in the intestine of the sea bream (*Sparus Aurata* L.). *Journal of Experimental Biology*. 216(3): 470-479. doi: <https://doi.org/10.1242/jeb.073742>.

Grentz L & Massey LK. 2002. Contribution of dietary oxalate to urinary oxalate in health and disease. *Topics in Clinical Nutrition*. 17(2): 60-70.

Grosell M. 2006. Intestinal anion exchange in marine fish osmoregulation. *Journal of Experimental Biology*. 209 (15): 2813–2827. doi: <https://doi.org/10.1242/jeb.02345>.

Grosell M, De-Boeck G, Johannsson O, Wood CM. 1999. The effects of silver on intestinal ion and acid-base regulation in the marine teleost fish, *Parophrys vetulus*. *Journal of Comparative Biochemistry and Physiology – Part C: Pharmacology, Toxicology and Endocrinology*. 124(3): 259-270. doi: 10.1016/s0742-8413(99)00073-0.

Grosell M & Genz J. 2006. Ouabain sensitive bicarbonate secretion and acidic fluid absorption by the marine teleost intestine play a role in osmoregulation. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*. 291(4): R1145-R1156. doi: <https://doi.org/10.1152/ajpregu.00818.2005>.

Grosell M & Jensen FB. 1999. NO₂ uptake and HCO₃⁻ secretion in the intestine of the European flounder (*Plactichthys flesus*). *Journal of Experimental Biology*. 202(15): 2103-2110. doi: 10.1242/jeb.202.15.2103.

Grosell M, Laliberte CN, Wood S, Jensen FB, Wood CM. 2001. Intestinal HCO₃⁻ secretion in marine teleost fish: evidence for an apical rather than a basolateral Cl⁻/HCO₃⁻ exchanger. *Fish Physiology and Biochemistry*. 24(2): 81-95. doi: <https://doi.org/10.1023/A:1011994129743>.

Grosell M, Mager EM, Williams C, Taylor JR. 2009. High rates of HCO₃⁻ secretion and Cl⁻ absorption against adverse gradients in the marine teleost intestine: the involvement of an electrogenic anion exchanger and H⁺-pump metabolon?. *Journal of Experimental Biology*. 212(11): 1684-1696. doi: <https://doi.org/10.1242/jeb.027730>.

Grosell M, Schauer KL, Christensen EAF. 2018. Comparison of the organic matrix found in intestinal CaCO₃ precipitates produced by several marine teleost species. *Journal of Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*. 221(11): 15-23. doi: 10.1016/j.cbpa.2018.03.007.

Grosell M & Wilson RW. 2003. Intestinal bicarbonate secretion in marine teleost fish—source of bicarbonate, pH sensitivity, and consequences for whole animal acid–base and calcium homeostasis. *Biochimica Et Biophysica Acta (BBA) – Biomembranes*. 1618(2): 163-174. doi:10.1016/j.bbamem.2003.09.014.

Hardy RW. 2010. Utilization of plant proteins in fish diets: effects of global demand and supplies of fishmeal. *Journal of Aquaculture Research*. 41(5): 770-776. doi: <https://doi.org/10.1111/j.1365-2109.2009.02349.x>.

Hatch M & Vaziri ND. 1994. Enhanced enteric excretion of urate in rats with chronic renal failure. *Clinical Science*. 86(5): 511-516. doi:10.1042/cs0860511.

Hatch M, Cornelius J, Allison M, Sidhu H, Peck A, Freel RW. 2006. *Oxalobacter* sp. reduces urinary oxalate excretion by promoting enteric oxalate secretion. *Kidney International*. 69(4): 691-698. doi: 10.1038/sj.ki.5000162.

Hatch M, Freel RW, Vaziri ND. 1994. Intestinal excretion of oxalate in chronic renal failure. *Journal of the American Society of Nephrology*. 5(6): 1339-1343. doi: <https://doi.org/10.1681/ASN.V561339>.

Hatch M, Gjymishka A, Salido EC, Allison MJ, Freel RW. 2011. Enteric oxalate elimination is induced and oxalate is normalized in a mouse model of primary hyperoxaluria following intestinal colonization with *Oxalobacter*. *American Journal of Physiology, Gastrointestinal and Liver Physiology*. 300(3), G461-G469. doi: <https://doi.org/10.1152/ajpgi.00434.2010>.

Hixson SM. 2014. Fish nutrition and current issues in aquaculture: the balance in providing safe and nutritious seafood, in an environmentally sustainable manner. *Journal of Aquaculture Research and Development*. 5(3): 2155-9546. doi: <http://dx.doi.org/10.4172/2155-9546.1000234>.

Holmes RP, Ambrosius WT, Assimos DG. 2005. Dietary oxalate loads and renal oxalate handling. *The Journal of Urology*. 174(3): 943-947. doi: <https://doi.org/10.1097/01.ju.0000169476.85935.e2>.

Holmes RP, Goodman HO, Assimos DG. 2001. Contribution of dietary oxalate to urinary oxalate excretion. *Kidney International*. 59(1): 270-276. doi:10.1046/j.1523-1755.2001.00488.x.

Huang Y, Zhang YH, Chi ZP, Huang R, Huang H, Liu G, Zhang Y, Yang H, Lin J, Yang T, Cao SZ. 2020. The handling of oxalate in the body and the origin of oxalate in calcium oxalate stones. *Urologia Internationalis*. 104(3-4): 167-176. doi:10.1159/000504417.

Hwang P, Lee T, Lin Y. 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 301(1): 28-47. doi:10.1152/ajpregu.00047.2011.

Hvizdak J. "Sailfin molly - *Poecilia latipinna*." *A Guide on Caring for Sailfin Mollies (Poecilia latipinna)*. Aqua-Fish Net. 19 June 2014. en.aqua-fish.net/fish/sailfin-molly.

Jacob P, Rossmann H, Lamprecht G, Kretz A, Neff C, Lin-Wu E, Gregor M, Groneberg DA, Kere J, Seidler U. 2002. Down-regulated in adenoma mediates apical Cl⁻/HCO₃⁻ exchange in rabbit, rat, and human duodenum. *Gastroenterology*. 122(3): P709-P724. doi: <https://doi.org/10.1053/gast.2002.31875>.

Jiang Z, Asplin JR, Evan AP, Rajendran VM. 2006. Calcium oxalate urolithiasis in mice lacking anion transporter SLC26A6. *Nature Genetics*. 38(4): 474-478. doi: <https://doi.org/10.1038/ng1762>.

Jiang Z, Grichtchenko II, Boron WF, Aronson PS. 2002. Specificity of anion exchange mediated by mouse SLC26A6. *Journal of Biological Chemistry*. 277(37): 33963-33967. doi: <https://doi.org/10.1074/jbc.m202660200>.

Kato A, Chang M, Kurita Y, Nakada T, Ogoshi M, Nakazato T, Doi H, Hirose S, Romero MF. 2009. Identification of renal transporters involved in sulfate excretion in marine teleost fish. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 297(6): R1647–R1659. doi: <https://doi.org/10.1152/ajpregu.00228.2009>.

Kaufman DW, Kelly JP, Curhan GC, Anderson TE, Dretler SP, Preminger GM, Cave DR. 2008. *Oxalobacter formigenes* may reduce the risk of calcium oxalate kidney stones. *Journal of the American Society of Nephrology*. 19(6): 1197-1203. doi: 10.1681/ASN.2007101058.

Khamaysi A, Anbtawee-Jomaa S, Fremder M, Eini-Rider H, Shimshilashvili L, Aharon S, Aizenshtein E, Shlomi T, Noguchi A, Springer D, Moe OW, Shcheynikov N, Muallem S, Ohana E. 2019. Systemic succinate homeostasis and local succinate signaling affect blood pressure and modify risks for calcium oxalate lithogenesis. *Journal of the American Society of Nephrology*. 30(3): 381-392. doi: <https://doi.org/10.1681/asn.2018030277>.

Kultz D. 2015. Physiological mechanisms used by fish to cope with salinity stress. *Journal of Experimental Biology*. 218(12): 1907–1914. doi:10.1242/jeb.118695.

Knauf F, Yang C, Thomson RB, Aronson PS. 2001. Identification of a chloride-formate exchanger expressed on the brush border membrane of renal proximal tubule cells. *Proceedings of the National Academy of Sciences*. 98(16): 9425-9430. doi: <https://doi.org/10.1073/pnas.141241098>.

Knauf F, Thomson RB, Heneghan JF, Jiang Z, Adebamiro A, Thomson CL, Barone C, Asplin JR, Egan ME, Alper SL, Aronson PS. 2017. Loss of cystic fibrosis transmembrane regulator impairs intestinal oxalate secretion. *Journal of the American Society of Nephrology*. 28(1): 242-249. doi: <https://doi.org/10.1681/asn.2016030279>.

Knight J, Assimios DG, Callahan MF, Holmes RP. 2011. Metabolism of primed, constant infusions of [1,2-¹³C₂] glycine and [1-¹³C₁] phenylalanine to urinary oxalate. *Metabolism*. 60(7): 950-956. doi: 10.1016/j.metabol.2010.09.002.

Knight J, Deora R, Assimios DG, Holmes RP. 2013. The genetic composition of *Oxalobacter formigenes* and its relationship to colonization and calcium oxalate stone disease. *Urolithiasis*. 41(3): 187-196. doi: <https://doi.org/10.1007/s00240-013-0566-7>.

Kormas KA, Meziti A, Mente E, Frentzos A. 2014. Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*). *Microbiology Open*. 3(5): 718-728. doi: <https://doi.org/10.1002/mbo3.202>.

- Kurita Y, Nakada T, Kato A, Doi H, Mistry AC, Chang M, Romero MF, Hirose S. 2008. Identification of intestinal bicarbonate transporters involved in formation of carbonate precipitates to stimulate water absorption in marine teleost fish. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*. 294(4): R1402–R1412. doi: <https://doi.org/10.1152/ajpregu.00759.2007>.
- Lange JN, Wood KD, Wong H, Otto R, Mufarrij PW, Knight J, Akpinar H, Holmes RP, Assimios DG. 2012. Sensitivity of human strains of *Oxalobacter formigenes* to commonly prescribed antibiotics. *Urology*. 79(6): 1286-1289. doi: 10.1016/j.urology.2011.11.017.
- Larsen EH, Deaton LE, Onken H, O'Donnell M, Grosell M, Dantzler WH, Weihrauch D. 2014. Osmoregulation and excretion. *Journal of Comprehensive Physiology*. 4(2): 405-573. doi: <https://doi.org/10.1002/cphy.c130004>.
- Li Q. “Solutions set in stone: an innovative, nutrition-based approach to managing kidney stones.” Research Publishing International Ltd. *Research Features*. 1 Feb. 2017. researchfeatures.com/managing-kidney-stones/.
- Lindsjo M, Danielson BG, Fellstrom B, Ljunghall S. 1989. Intestinal oxalate and calcium absorption in recurrent renal stone formers and healthy subjects. *Scandinavian Journal of Urology and Nephrology*. 23(1): 55-59. doi: 10.1080/00365599.1989.11690431.
- Liu Y, Jin X, Ma Y, Jian Z, Wei Z, Xiang L, Sun Q, Qi S, Wang K, Li H. 2021. Short-chain fatty acids reduced renal calcium oxalate stones by regulating the expression of intestinal oxalate transporter SLC26A6. *American Society for Microbiology*. 6(6): e01045-21. doi: <https://doi.org/10.1128/msystems.01045-21>.
- Marshall WS & Grosell M. 2006. Ion transport, osmoregulation and acid-base balance. In *Physiology of Fishes*. Volume 3 (ed. D. Evans and J. B. Claiborne). pp. 177-230. Boca Raton: CRC Press.
- Martin L & Esbaugh AJ. 2021. Osmoregulatory plasticity during hypersaline acclimation in red drum, *Sciaenops ocellatus*. *Journal of Comparative Physiology – Part B: Biochemistry and Molecular Biology*. 191: 731-740. doi: <https://doi.org/10.1007/s00360-021-01356-y>.
- Mehta M, Goldfarb DS, Nazzal L. 2016. The role of the microbiome in kidney stone formation. *International Journal of Surgery*. 36(Part D): 607-612. doi: 10.1016/j.ijssu.2016.11.024.
- Massey LK, Palmer RG, Horner HT. 2001. Oxalate content of soybean seeds (*Glycine max*: leguminosae), soyfoods, and other edible legumes. *Journal of Agricultural and Food Chemistry*. 49(9): 4262-4266. doi: <https://doi.org/10.1021/jf010484y>.
- Miller A & Dearing MD. 2013. The metabolic and ecological interactions of oxalate-degrading bacteria in the mammalian gut. *Pathogens*. 2(4): 636-652. doi:10.3390/pathogens2040636.

Miller A, Choy D, Penniston K, Lange D. 2019. Inhibition of urinary stone disease by a multi-species bacterial network ensures healthy oxalate homeostasis. *Kidney International*. 96(1): 180-188. doi: 10.1016/j.kint.2019.02.012.

Miller A, Kohl K, Dearing MD. 2014. The gastrointestinal tract of the white-throated woodrat (*Neotoma albigula*) harbors distinct consortia of oxalate-degrading bacteria. *Applied and Environmental Microbiology*. 80(5): 1595-1601. doi: 10.1128/AEM.03742-13.

Miller AW, Orr T, Dearing D, Monga M. 2019. Loss of function dysbiosis associated with antibiotics and high fat, high sugar diet. *Multidisciplinary Journal of Microbial Ecology*. 13(6): 1379-1390. doi: 10.1038/s41396-019-0357-4.

Molnar C & Gair J. “22.1 Osmoregulation and osmotic balance.” *Concepts of Biology 1st Canadian Edition*. 1 May 2019. opentextbc.ca/biology/chapter/22-1-osmoregulation-and-osmotic-balance/.

Mount DB & Romero MF. 2003. The SLC26 gene family of multifunctional anion exchangers. *Pflügers Archiv - European Journal of Physiology*. 447(5): 710-721. doi: <https://doi.org/10.1007/s00424-003-1090-3>.

Mukaibo T, Munemasa T, George AT, Shull GE, Soleimani M, Melvin JE. 2018. The apical anion exchanger SLC26A6 promotes oxalate secretion by murine submandibular gland acinar cells. *Journal of Biological Chemistry*. 293(17): 6259–6268. doi: <https://doi.org/10.1074/jbc.ra118.002378>.

Murawala H, Patel S, Ranadive I, Desai I, Balakrishnan S. 2018. Variation in expression and activity pattern of mmp2 and mmp9 on different time scales in the regenerating caudal fin of *Poecilia latipinna*. *Journal of Fish Biology*. 92(5): 1604–1619. doi: <https://doi.org/10.1111/jfb.13618>.

Naylor RL, Goldberg RJ, Primavera JH, Kautsky N, Beveridge MC, Clay J, Folke C, Lubchenco J, Mooney H, Troell M. 2000. Effect of aquaculture on world fish supplies. *Nature*. 405: 1017-1024. doi: <https://doi.org/10.1038/35016500>.

Nazzari L & Blaser MJ. 2018. Does the receipt of antibiotics for common infectious diseases predispose to kidney stones? A cautionary note for all health care practitioners. *Journal of the American Society of Nephrology*. 29(6): 1590-1592. doi: <https://doi.org/10.1681/ASN.2018040402>.

Neumeier LI, Thomson RB, Reichel M, Eckardt KU, Aronson PS, Knauf F. 2020. Enteric oxalate secretion mediated by SLC26A6 defends against hyperoxalemia in murine models of chronic kidney disease. *Journal of the American Society of Nephrology*. 31(9): 1987-1995. doi: <https://doi.org/10.1681/ASN.2020010105>.

Nour AMA & Pfaffl MW. 2020. “Inhomogenous replicates.” In *MIQE & qPCR: how to apply the MIQE guidelines: a visual, interactive and practical qPCR guide!* (4th Edition). pp. 475. ISBN: 9783000488061.

Nour AMA & Pfaffl MW. 2020. “PCR amplification efficiency.” In *MIQE & qPCR: how to apply the MIQE Guidelines: a visual, interactive and practical qPCR guide!* (4th Edition). pp. 214. ISBN: 9783000488061.

Nozawa T, Sugiura S, Hashino Y, Tsuji A, Tamai I. 2004. Role of anion exchange transporter PAT1 (SLC26A6) in intestinal absorption of organic anions. *Journal of Drug Targeting*. 12 (2): 97–104. doi: <https://doi.org/10.1080/10611860410001693742>.

Perinpan M, Enders FT, Mara KC, Vaughan LE, Mehta RA, Voskoboev N, Milliner DS, Lieske JC. 2017. Plasma oxalate in relation to eGFR in patients with primary hyperoxaluria, enteric hyperoxaluria and urinary stone disease. *Journal of Clinical Biochemistry*. 50(18): 1014-1019. doi: [10.1016/j.clinbiochem.2017.07.017](https://doi.org/10.1016/j.clinbiochem.2017.07.017).

Prenen JA, Boer P, Dorhout-Mees EJ. 1984. Absorption kinetics of oxalate from oxalate-rich food in man. *American Journal of Clinical Nutrition*. 40(5): 1007-1010. doi: [10.1093/ajcn/40.5.1007](https://doi.org/10.1093/ajcn/40.5.1007).

Rajaram S, Murawala H, Buch P, Patel S. 2016. Inhibition of BMP signaling reduces mmp-2 and mmp-9 expression and obstructs wound healing in regenerating fin of teleost fish *Poecilia latipinna*. *Fish Physiology and Biochemistry*. 42(2): 787–794. doi: <https://doi.org/10.1007/s10695-015-0175-1>.

Rakocy JE. “*Oreochromis niloticus* – cultured aquatic species information programme.” *FAO (Food and Agriculture Organization of the United Nations) Fisheries and Aquaculture Division*. 19 May 2006. https://www.fao.org/fishery/en/culturedspecies/Oreochromis_niloticus/en.

Robijn S, Hoppe B, Vervaeke BA, D’Haese PC, Verhulst A. 2011. Hyperoxaluria: a gut–kidney axis?. *Kidney International*. 80(11): 1146-1158. doi: <https://doi.org/10.1038/ki.2011.287>.

Ruhr IM, Takei Y, Grosell M. 2016. The role of the rectum in osmoregulation and the potential effect of renoguanin on SLC26A6 transport activity in the Gulf toadfish (*Opsanus beta*). *American Journal of Physiology – Regulatory, Integrative and Comparative Physiology*. 311(1): R179-R191. doi: <https://doi.org/10.1152/ajpregu.00033.2016>.

Ruiz-Jarabo I, Gregorio SF, Gaetano P, Trischitta F, Fuentes J. 2017. High rates of intestinal bicarbonate secretion in seawater tilapia (*Oreochromis mossambicus*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*. 207; 57-64. doi: <https://doi.org/10.1016/j.cbpa.2017.02.022>.

Sayer JA. 2017. Progress in understanding the genetics of calcium-containing nephrolithiasis. *Journal of the American Society of Nephrology*. 28(3): 748-759. doi: <https://doi.org/10.1681/ASN.2016050576>.

Schmidt V, Smith KF, Melvin DW, Amaral-Zettler LA. 2015. Community assembly of a euryhaline fish microbiome during salinity acclimation. *Molecular Ecology*. 24(10): 2537-2550. doi: 10.1111/mec.13177.

Seidler U & Nikolovska K. 2019. SLC26 family of anion transporters in the gastrointestinal tract: expression, function, regulation, and role in disease. *Comprehensive Physiology*. 9(2): 839-872. doi: <https://doi.org/10.1002/cphy.c180027>.

Shah RJ, Vaughan LE, Enders FT, Milliner DS, Lieske JC. 2020. Plasma oxalate as a predictor of kidney function decline in a primary hyperoxaluria cohort. *International Journal of Molecular Sciences*. 21(10): e3608. doi: 10.3390/ijms21103608.

Shehadeh ZH & Gordon MS. 1969. Role of the intestine in salinity adaptation of the rainbow trout, *Salmo gairdneri*. *Journal of Comparative Biochemistry and Physiology*. 30(3): 397-418. doi: [https://doi.org/10.1016/0010-406X\(69\)92011-8](https://doi.org/10.1016/0010-406X(69)92011-8).

Sun Z, Li G, Wang C, Jing Y, Zhu Y, Zhang S, Liu Y. 2014. Community dynamics of prokaryotic and eukaryotic microbes in an estuary reservoir. *Scientific Reports*. 4(1): 6966. doi: <https://doi.org/10.1038/srep06966>.

Takei Y. 2021. The digestive tract as an essential organ for water acquisition in marine teleosts: lessons from euryhaline eels. *Zoological Letters*. 7(1): 10. doi: <https://doi.org/10.1186/s40851-021-00175-x>.

Takei Y, Wong MKS, Ando M. 2019. Molecular mechanisms for intestinal HCO₃⁻ secretion and its regulation by guanylin in seawater-acclimated eels. *Journal of Experimental Biology*. 222(13): jeb203539. doi: <https://doi.org/10.1242/jeb.203539>.

Tarnecki A, Burgos F, Ray C, Arias C. 2017. Fish intestinal microbiome: diversity and symbiosis unravelled by metagenomics. *Journal of Applied Microbiology*. 123(1): 2-17. doi: <https://doi.org/10.1111/jam.13415>.

Tasian GE, Jemielita T, Goldfarb DS, Copelovitch L, Gerber JS, Wu Q, Denburg MR. 2018. Oral antibiotic exposure and kidney stone disease. *Journal of the American Society of Nephrology*. 29(6): 1731-1740. doi: 10.1681/ASN.2017111213.

Taylor JR & Grosell M. 2006. Evolutionary aspects of intestinal bicarbonate secretion in fish. *Journal of Comparative Biochemistry and Physiology*. 143(A): 523-529. doi: 10.1016/j.cbpa.2006.01.027.

Taylor S, Wakem M, Dijkman G, Alsarraj M, Nguyen M. 2010. A practical approach to RT-qPCR-publishing data that conform to the MIQE guidelines. *BioRad Laboratories Inc*. 50(4): S1-S5. doi: 10.1016/j.ymeth.2010.01.005.

- Thomson RB, Wang T, Thomson BR, Tarrats L, Girardi A, Mentone S, Soleimani M, Kocher O, Aronson PS. 2005. Role of PDZK1 in membrane expression of renal brush border ion exchangers. *Proceedings of the National Academy of Sciences*. 102(37): 13331–13336. doi: <https://doi.org/10.1073/pnas.0506578102>.
- Vatsos IN. 2016. Standardizing the microbiota of fish used in research. *Lab Animal*. 51(4): 353-364. doi: [10.1177/0023677216678825](https://doi.org/10.1177/0023677216678825).
- Verdeyroux P. “Wels catfish monitoring.” *Continental Aquatic Ecosystems Network*. 19 Mar. 2019. <https://cae-mab-network.com/wels-catfish-monitoring/>.
- Walker LE. 1971. “Osmotic and ionic regulation in a fresh-water teleost, *Ictalurus nebulosus* (Le Sueur).” Ph.D. Thesis. *Oregon State University Theses*. pp. 1–82.
- Watanabe T & Takei Y. 2011. Molecular physiology and functional morphology of SO_4^{2-} excretion by the kidney of seawater-adapted eels. *Journal of Experimental Biology*. 214(10): 1783–1790. doi: <https://doi.org/10.1242/jeb.051789>.
- Wang J, Wang W, Wang H, Tuo B. 2021. Physiological and pathological functions of SLC26A6. *Frontiers in Medicine*. Volume 7. doi: <https://doi.org/10.3389/fmed.2020.618256>.
- Wang Z, Wang T, Petrovic S, Tuo B, Riederer B, Barone S, Lorenz J, Seidler U, Aronson P, Soleimani M. 2005. Renal and intestinal transport defects in SLC26A6-null mice. *American Journal of Physiology – Cell Physiology*. 288(4): C957-C965. doi: <https://doi.org/10.1152/ajpcell.00505.2004>.
- Whittamore JM. 2020. The teleost fish intestine is a major oxalate-secreting epithelium. *Journal of Experimental Biology*. 223(12): 553-558. doi: [10.1242/jeb.216895](https://doi.org/10.1242/jeb.216895).
- Whittamore JM & Hatch M. 2017. The role of intestinal oxalate transport in hyperoxaluria and the formation of kidney stones in animals and man. *Urolithiasis*. 45(1): 89–108. doi: [10.1007/s00240-016-0952-z](https://doi.org/10.1007/s00240-016-0952-z).
- Wilson RW. 1999. A novel role for the gut of seawater teleosts in acid-base balance. In *Regulation of Acid-Base Status in Animals and Plants, SEB Seminar Series* (Ed. Egginton S, Taylor EW, Raven JA). pp. 257-274. Cambridge: Cambridge University Press.
- Wilson RW, Gilmour KM, Henry RP, Wood CM. 1996. Intestinal base excretion in the seawater-adapted rainbow trout: a role in acid-base balance?. *Journal of Experimental Biology*. 199(10): 2331-2343. doi: [10.1242/jeb.199.10.2331](https://doi.org/10.1242/jeb.199.10.2331).
- Wilson RW & Grosell M. 2003. Intestinal bicarbonate secretion in marine teleost fish-source of bicarbonate, pH sensitivity, and consequences for whole animal acid-base and calcium homeostasis. *Biochimica Biophysica Acta Biomembranes*. 1618(2): 163-174. doi: <https://doi.org/10.1016/j.bbamem.2003.09.014>.

Wedenoja S, Ormala T, Berg UB, Halling SF, Jalanko H, Karikoski R, Kere J, Holmberg C, Hoglund P. 2008. The impact of sodium chloride and volume depletion in the chronic kidney disease of congenital chloride diarrhea. *Kidney International*. 74(8): 1085-1093. doi: <https://doi.org/10.1038/ki.2008.401>.

Wilson RW, Wilson JM, Grosell M. 2002. Intestinal bicarbonate secretion by marine teleost fish—why and how?. *Biochimica Biophysica Acta Biomembranes*. 1566(1-2): 182-193. doi: [https://doi.org/10.1016/S0005-2736\(02\)00600-4](https://doi.org/10.1016/S0005-2736(02)00600-4).

Williams AW & Wilson DM. 1990. Dietary intake, absorption, metabolism and excretion of oxalate. *Seminars in Nephrology*. 10(1): 2-8. ISSN: 0270-9295.

Xie Q, Welch R, Mercado A, Romero MF, Mount DB. 2002. Molecular characterization of the murine SLC26A6 anion exchanger: functional comparison with SLC26A1. *American Journal of Physiology-Renal Physiology*. 283(4): F826–F838. doi: <https://doi.org/10.1152/ajprenal.00079.2002>.

Zampini A, Nguyen AH, Rose E, Monga M, Miller AW. 2019. Defining dysbiosis in patients with urolithiasis. *Scientific Reports*. 9: 5425. doi: [10.1038/s41598-019-41977-6](https://doi.org/10.1038/s41598-019-41977-6).

Zhang M, Sun Y, Liu Y, Qiao F, Chen L, Liu W, Du Z, Li E. 2016. Response of gut microbiota to salinity change in two euryhaline aquatic animals with reverse salinity preference. *Aquaculture*. 454: 72-80. doi: <https://doi.org/10.1016/j.aquaculture.2015.12.014>.

Zhang X, Wen H, Wang H, Ren Y, Zhao J, Li Y. 2017. RNA-seq analysis of salinity stress-responsive transcriptome in the liver of spotted sea bass (*Lateolabrax maculatus*). *PLoS One*. 12(3): e0173238. doi: <https://doi.org/10.1371/journal.pone.0173238>.

Zhao R, Symonds JE, Walker SP, Steiner K, Carter CG, Bowman JP, Nowak BF. 2020. Salinity and fish age effect the gut microbiota of farmed Chinook salmon (*Oncorhynchus tshawytscha*). *Aquaculture*. 528. doi: <https://doi.org/10.1016/j.aquaculture.2020.735539>.

APPENDIX

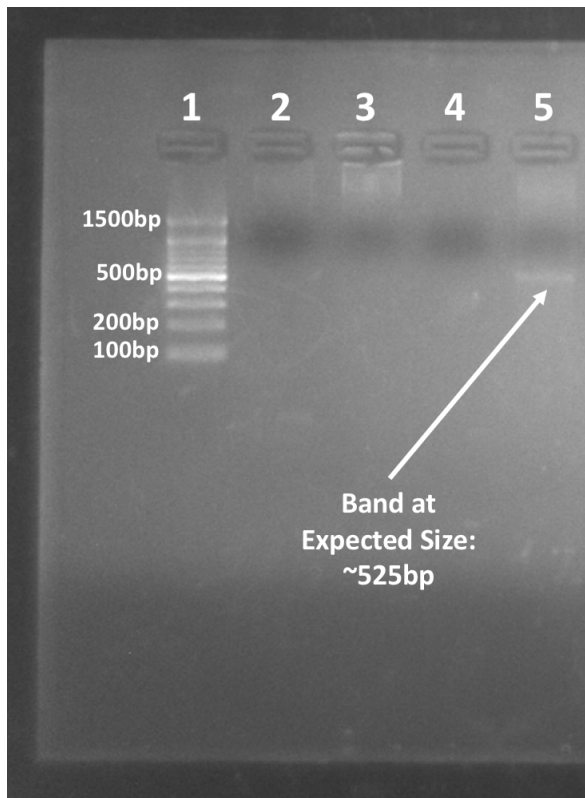


Figure 3.1: PCR products of gDNA extracted from intestinal tissues of sailfin mollies shown on 1.5% agarose gel electrophoresis (70V for 30 minutes) using universal bacterial rRNA primers 8F and 533R. gDNA was extracted from samples using Qiagen's *Soil Kit Pro*. The annealing temperature used was 55.5°C and PCR was run for 25 cycles.

Lane 1: O'RangeRuler 100bp DNA Ladder (Thermofisher Scientific); Lane 2: Genomic DNA from intestinal sample of SW-14 Day + Antibiotics fish; Lane 3: *Soil Kit Pro* negative control; Lane 4: PCR negative; Lane 5: gDNA from intestinal sample of FW fish (PCR positive).

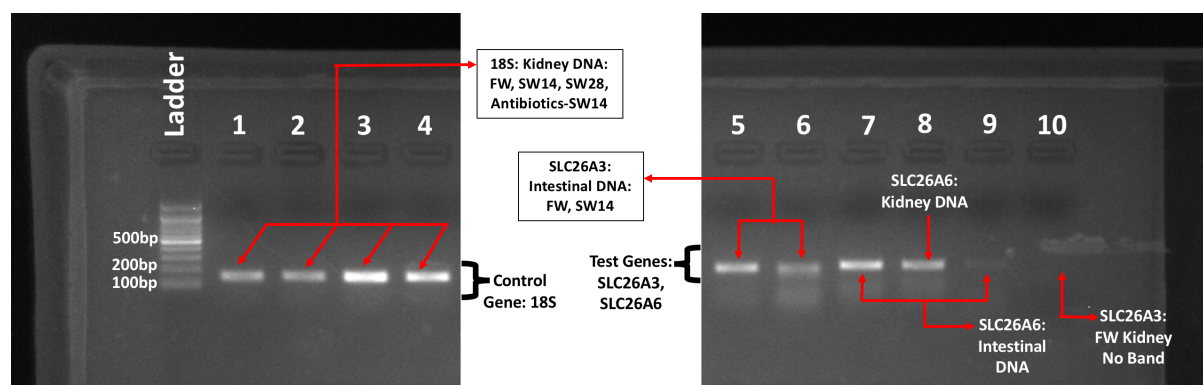


Figure 3.2A: Using gradient PCR to confirm the absence of SLC26A3 expression in the kidneys. 40 cycles of PCR were conducted and gel electrophoresis was run on a 1.5% agarose (w/v) gel stained with ethidium bromide at 75 volts for 30 minutes. The DNA ladder used was the O'RangeRuler 100bp DNA Ladder (Thermofisher Scientific). Primers for the 18S gene were used as the positive control on kidney cDNA from all four conditions (FW, SW-14 Day, SW-28 Day, SW-14 Day + Antibiotics). SLC26A6 primers successfully yielded products when used on both intestinal and kidney cDNA. There was no product when PCR was conducted using the SLC26A3 primers on kidney cDNA from the FW condition. Please see Figure 3.2B for continuation of gradient PCR conducted on kidney cDNA using the SLC26A3 primers.

Lane 1: 18S Primers – FW Kidney (~100ng of cDNA) Annealing Temperature: 59.9°C;
 Lane 2: 18S Primers – SW-14 Day Kidney (~100ng of cDNA) Annealing Temperature: 60.5°C;
 Lane 3: 18S Primers – SW-28 Day Kidney (~100ng of cDNA) Annealing Temperature: 59.9°C;
 Lane 4: 18S Primers – SW-14 Day + Antibiotics Kidney (~100ng of cDNA) Annealing Temperature: 60.5°C;
 Lane 5: SLC26A3 Primers – FW Intestine (~100ng of cDNA) Annealing Temperature: 59.9°C;
 Lane 6: SLC26A3 Primers – SW-14 Day Intestine (~100ng of cDNA) Annealing Temperature: 60.5°C;
 Lane 7: SLC26A6 Primers – FW Intestine (~100ng of cDNA) Annealing Temperature: 59.9°C;
 Lane 8: SLC26A6 Primers – SW-14 Day Kidney (~100ng of cDNA) Annealing Temperature: 60.5°C;
 Lane 9: SLC26A6 Primers– SW-14 Day + Antibiotics Intestine (~100ng of cDNA) Annealing Temperature: 59.9°C;
 Lane 10: SLC26A3 Primers – FW Kidney (~100ng of cDNA) Annealing Temperature: 55.1°C.

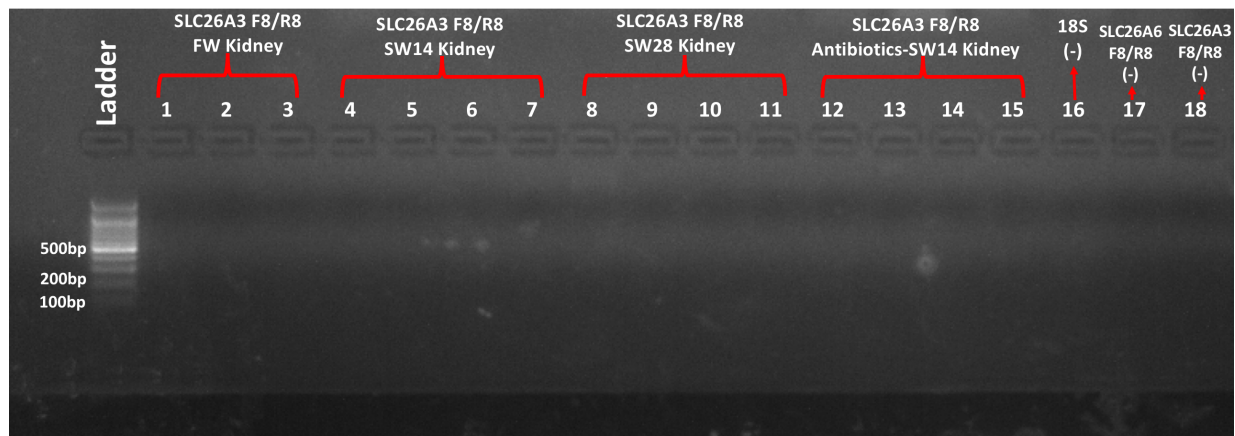


Figure 3.2B: Using gradient PCR to confirm the absence of SLC26A3 expression in the kidneys. 40 cycles of PCR were conducted and gel electrophoresis was run on a 1.5% agarose (w/v) gel stained with ethidium bromide at 75 volts for 30 minutes. The DNA ladder used was the O'RangeRuler 100bp DNA Ladder (Thermofisher Scientific). This gel image is a continuation of the gradient PCR shown in Figure 3.2A. There were no products yielded when PCR was conducted with the SLC26A3 primers on kidney cDNA from any of the four conditions (FW, SW-14 Day, SW-28 Day, SW-14 Day + Antibiotics). The negative controls for 18S, SLC26A6 and SLC26A3 are also shown in this gel image.

Lane 1: SLC26A3 Primers – FW Kidney (~100ng of cDNA) Annealing Temperature: 56.0°C;
 Lane 2: SLC26A3 Primers – FW Kidney (~100ng of cDNA) Annealing Temperature: 57.4°C;
 Lane 3: SLC26A3 Primers – FW Kidney (~100ng of cDNA) Annealing Temperature: 59.1°C;
 Lane 4: SLC26A3 Primers – SW-14 Day Kidney (~100ng of cDNA) Annealing Temperature: 55.1°C;
 Lane 5: SLC26A3 Primers – SW-14 Day Kidney (~100ng of cDNA) Annealing Temperature: 56.0°C;
 Lane 6: SLC26A3 Primers – SW-14 Day Kidney (~100ng of cDNA) Annealing Temperature: 57.4°C;
 Lane 7: SLC26A3 Primers – SW-14 Day Kidney (~100ng of cDNA) Annealing Temperature: 59.1°C;
 Lane 8: SLC26A3 Primers – SW-28 Day Kidney (~100ng of cDNA) Annealing Temperature: 55.1°C;
 Lane 9: SLC26A3 Primers – SW-28 Day Kidney (~100ng of cDNA) Annealing Temperature: 56.0°C;
 Lane 10: SLC26A3 Primers – SW-28 Day Kidney (~100ng of cDNA) Annealing Temperature: 57.4°C;
 Lane 11: SLC26A3 Primers – SW-28 Day Kidney (~100ng of cDNA) Annealing Temperature: 59.1°C;
 Lane 12: SLC26A3 Primers – SW-14 Day + Antibiotics Kidney (~100ng of cDNA) Annealing Temperature: 55.1°C;
 Lane 13: SLC26A3 Primers – SW-14 Day + Antibiotics Kidney (~100ng of cDNA) Annealing Temperature: 56.0°C;
 Lane 14: SLC26A3 Primers – SW-14 Day + Antibiotics Kidney (~100ng of cDNA) Annealing Temperature: 57.4°C;
 Lane 15: SLC26A3 Primers – SW-14 Day + Antibiotics Kidney (~100ng of cDNA) Annealing Temperature: 59.1°C;
 Lane 16: 18S Primers – Negative Control: Water – Annealing Temperature: 59.9°C;
 Lane 17: SLC26A6 Primers – Negative Control: Water – Annealing Temperature: 59.9°C;
 Lane 18: SLC26A3 Primers – Negative Control: Water – Annealing Temperature: 59.9°C.

Table 3.1: Product sequences of the SLC26A3 and SLC26A6 primer sets designed for the sailfin molly

Primer Set	Product Sequence (5' → 3')	Product Length
SLC26A3	CGGTACAAGAAAGTACAGGGGGGAAAACTCAG ATCGCTGGTTTACTATCAGCTGTTATAGTGATGA TTGTCACCTTTGGCTATTGGATATCTTCTTGAGCCT TTACCAAAGTCTGTACTGGGTGCCGTGGTCATCA TCAACCTGAAGGGCATGCTGATGCAGTTCAATGA AATTCCTTACCTGTGGAGGAGAGACAGGCCAGA TTGTGTGGTGTGGA	216bp
SLC26A6	TCCAGTGTTTCGCCATCAGCTGCTCAATGTCTC GCAGTTTGGTTCAGGAAAGCACCGGAGGAAAAA CCCAGATTGCTGGGGCTCTATCGGCGGTGGTTAT CCTTTTCATCACTCTCTGGATTGGATCTCTCTTTG AAGATCTACCCAAGGCAGTGCTGGCTGCCATCAT CTTCGTTAATCTCCATGGCATGATGAAGCAGTTC TTGGACATCCCCGCTCTGTGGAAAAGCAAT	233bp

*The sequences in bold are the forward and reverse primers.