

**Impact of nitrite on the molecular physiology of the guppy
(*Poecilia reticulata* Peters, 1859) gill epithelium tight junction complex**

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Abstract

Nitrite (NO_2^-) levels in natural freshwater (FW) are typically low, but anthropogenic activity can elevate them, impairing osmoregulatory mechanisms in FW teleost gills. While transcellular disruption by NO_2^- is documented, its impact on paracellular osmoregulation via the gill tight junction (TJ) complex remains unexplored. This study examined how sublethal waterborne NO_2^- exposure (7 days) affects osmoregulation in FW guppies (*Poecilia reticulata*), focusing on the molecular physiology of the gill TJ complex. Gills were analyzed for TJ protein gene transcript abundance, Na^+, K^+ -ATPase activity, and ultrastructural changes. Elevated NO_2^- reduced carcass Na^+ and Cl^- levels and gill Na^+, K^+ -ATPase activity, indicating osmoregulatory imbalance. TJ gene transcript abundance was unaffected except *claudin-10e*, which decreased with NO_2^- , suggesting reduced mitochondria-rich cells (MRCs). Ultrastructural changes in MRCs revealed transcellular dysfunction (e.g., fewer mitochondria, tubular vacuolization) and compromised paracellular integrity (e.g., shallow junctions). Findings concur that NO_2^- primarily disrupts osmoregulation through transcellular pathways, with limited paracellular effects.

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List of Abbreviations

ACTB	β -Actin
ASIC	acid-sensing ion channel
Cl ⁻	chloride ion
Cl ⁻ /HCO ₃ ⁻	chloride-bicarbonate anion exchanger
CLC-2	chloride channel 2
Cldn	claudin
FW	freshwater
GI	gastrointestinal
GOI	gene of interest
H ⁺	proton
H ⁺ pump	vacuolar type H ⁺ -ATPase
HCO ₃ ⁻	bicarbonate
HKG	housekeeping gene
HNO ₃	nitric acid
IBD	inflammatory bowel disease
IPW	ion-poor water
ISME	ion selective microelectrode
K ⁺	potassium ion
LC50	lethal concentration 50
MRC	mitochondria-rich cell
N	nitrogen
N ₂	nitrogen gas
Na ⁺	sodium ion
Na,K-ATPase	sodium-potassium ATPase
NaNO ₂	sodium nitrite
NB	nitrifying bacteria
NH ₃	ammonia
NO	nitrogen monoxide
NO ₂ ⁻	nitrite
NO ₃ ⁻	nitrate
Ocln	occludin
PVC	pavement cell
RBC	red blood cell
RPL13A	ribosomal protein L13a
SW	saltwater
TEM	transmission electron microscopy
TJ	tight junction
TRIC	tricellulin
TUBA1B	tubulin alpha-1B chain
ZO1-3	zonula occludens proteins 1-3
18S rRNA	18S ribosomal RNA

1 Introduction

1.1 Overview

My thesis will consider the impact of nitrite (NO_2^-) on freshwater (FW) teleost fishes with an emphasis on osmoregulation as well as gill epithelium function. The nitrogen (N) cycle is a critical process for the survival of all organisms, both in terrestrial and aquatic settings. However, natural and/or anthropogenic alteration of the N cycle can lead to the build-up of harmful nitrogenous waste such as NO_2^- .

1.2 NO_2^- in the Environment

N is a structural component of all amino acids, and therefore, many proteins. While nitrogen gas (N_2) is the main component of Earth's atmosphere, N is inaccessible to most organisms, since N_2 forms a strong (triple covalent) bond and must be converted to a reactive form. Reactive forms of N include ammonia (NH_3), ammonium, urea, nitrate (NO_3^-), NO_2^- , and various N oxide gases (Edwards et al., 2023). However, NH_3 is the central molecular component of the N cycle - a biogeochemical process that converts N to a reactive form by transiting N through the atmosphere, land, water, plants, animals, and microorganisms (Bernhard, 2010). The N cycle consists of several stages: fixation, which converts N_2 to NH_3 ; nitrification, which converts NH_3 to NO_3^- ; and denitrification, which converts NO_3^- to N_2 (Fig. 1). Nitrification can take place in soil or natural waters and is carried out by the nitrifying bacteria (NB) *Nitrosomonas* spp., which oxidize NH_3 to NO_2^- , and *Nitrobacter* spp., which oxidize NO_2^- to NO_3^- . However, anthropogenic input to FW systems - predominantly as NH_3 from fertilizer runoff but also as NO_2^- from sodium nitrite (NaNO_2) industrial waste (e.g., corrosion inhibitor manufacturing) (Fawell et al., 2017) - can impair these bacteria.

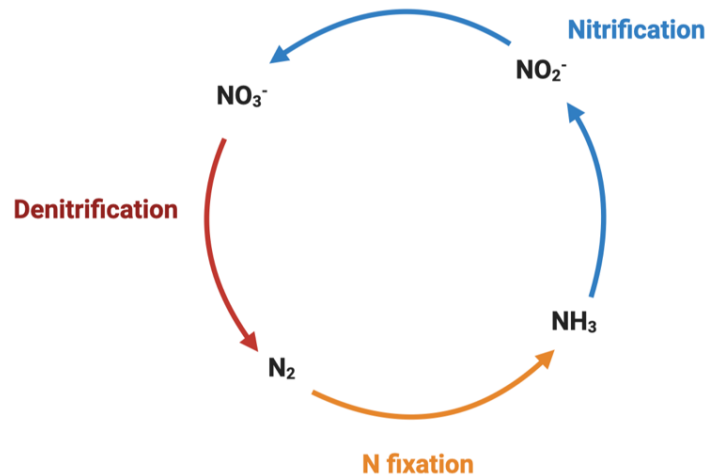


Figure 1: The nitrogen (N) cycle.

A biogeophysical process, crucial to the survival of all living organisms, dependent on the action of select microbes to convert nitrogen gas (N_2) to ammonia (NH_3) (fixation), NH_3 to nitrate (NO_3^-) (nitrification), and NO_3^- to N_2 (denitrification) [adapted from (Bernhard, 2010); created with BioRender.com].

Anthropogenic altering of the N cycle is a major problem for aquatic systems, as it can lead to the eutrophication or acidification of natural waters (Vitousek et al., 1997; Bernhard, 2010). Eutrophication is harmful to NB, which are obligate aerobes, since it can result in algal blooms that deplete natural waters of oxygen. One of the mechanisms for natural FW acidification is the chemical reaction of NH_3 oxidation, which yields protons (H^+), carried out by *Nitrosomonsa* (Bernhard, 2010; Vitousek et al., 1997). Blackburne et al. (2007) studied the effect of pH on NB and noted that *Nitrobacter* activity is maximal at approximately pH 7.8 while relative activity degrades rapidly away from that pH (Fig. 2). Thus, anthropogenic input of NH_3 can impair *Nitrobacter* activity and lead to increased NO_2^- levels, which has several detrimental physiological effects on fish.

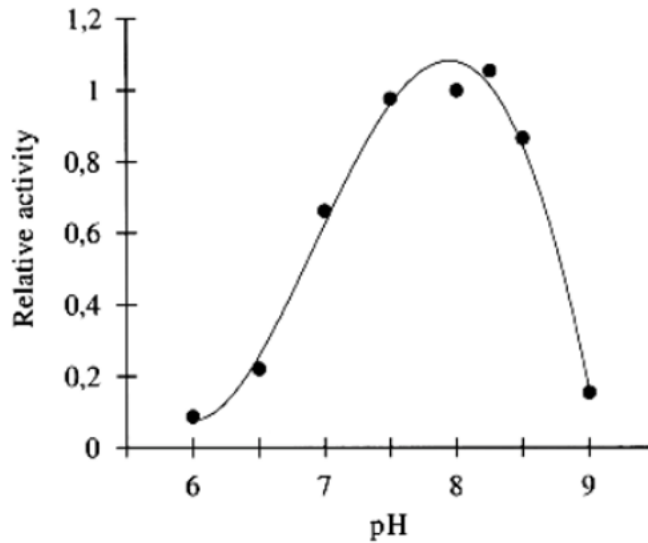


Figure 2: *Nitrobacter* pure culture relative activity dependence on pH.

Nitrobacter relative activity quickly degrades away from the optimal pH of 7.8 [adapted from (Blackburne et al., 2007)].

1.3 Deleterious Physiological Effects of NO_2^- on Fishes

The deleterious effects of NO_2^- on the physiology of fishes has been examined in several species: rainbow trout (*Oncorhynchus mykiss*) and perch (*Perca fluviatilis*) (Williams and Eddy, 1986; Jensen, 1993; Kroupova et al., 2008); carp (*Cyprinus carpio*) (Knudsen and Jensen, 1997; Svobodova et al., 2005). For example, entry of NO_2^- into red blood cells (RBCs) in carp is believed to be by conductive transport and to occur predominantly in deoxygenated cells (Jensen, 2003). As noted by Svobodova et al., after an acute exposure to NO_2^- , a major detrimental effect of NO_2^- build-up in RBCs of carp was the oxidation of hemoglobin to methemoglobin, the latter of which does not bind oxygen and leads to hypoxia, which causes hyperventilation and ultimately, organ damage (Kroupova et al., 2005). In RBCs, methemoglobin activates a potassium chloride (K^+/Cl^-) cotransporter, leading to elevated levels of extracellular potassium ions (K^+) and the drawing of osmotically obligated water out of the cells. Increased extracellular

K^+ is unfavourable for excitable tissues, such as heart pacemaker cells, since it reduces resting membrane potential, resulting in low conduction and decreased excitability that may lead to heart failure (Surawicz and Gettes, 1963). NO_2^- induced efflux of K^+ from skeletal muscles has been observed in carp and is believed to be via a transport route comparable to RBCs, since K^+ loss was accompanied by chloride ion (Cl^-) loss and reduced muscle water content, but the precise mechanism has not been investigated (Knudsen and Jensen, 1997).

As a further example, Kroupova et al. (2008) considered the effect, on the physiology of rainbow trout, from sub-chronic NO_2^- exposure over a 28-day period. A significantly lower growth rate was noted for the treatment group of fish exposed to the highest NO_2^- concentration ($3 \mu g/ml$) while all treatment groups displayed significantly higher glucose concentrations in blood plasma. Hyperglycemia is indicative of an elevated metabolism, due to increased energy demand from stress, suggesting that even low levels of NO_2^- can cause significant stress on fish.

Eddy and Williams (1987) indicated that NO_2^- levels up to $2 \mu g/l$ $N \cdot NO_2^-$ (where $N \cdot NO_2^-$ denotes mass N in NO_2^-) occur in unpolluted surface FW while NO_2^- levels in polluted FW can be several orders of magnitude greater (Table 1). This is problematic because elevated NO_2^- levels can be toxic to fish, and sublethal levels are documented to disrupt salt and water balance (Jensen, 2003).

Table 1: Nitrite (NO₂⁻) levels in natural freshwater.

Representative NO₂⁻ levels in waters receiving industrial wastewater effluents, sewage, and agricultural runoff.

Type of pollutant	NO ₂ ⁻ levels (mg/l N·NO ₂ ⁻)	Reference
Rivers receiving effluents from metal works	up to 30	Klinger, 1957
River Hume, West Yorkshire, sewage, textile works	1.2	Walsh et al., 1980
Mine and agricultural wastes	0.82	Cabrera et al., 1984

1.4 Osmoregulation in FW Teleost Fishes

FW teleosts are hyperosmotic to their environment: in contrast to FW osmolarity, which is about 0-1 mOsm, FW teleost blood plasma osmolarity is approximately 274 mOsm (Evans et al., 2005). Given the resulting inward osmotic and outward ionic gradients, FW teleosts face the problem of water loading and passive ion loss, particularly sodium ions (Na⁺) and Cl⁻, which are the dominant solutes (Table 2) (Evans et al., 2005).

Table 2: Representative osmolarity and major solute composition of freshwater (FW) teleost blood plasma.

FW teleost solute concentration is two orders of magnitude greater than FW and is dominated by sodium ions (Na⁺) and chloride ions (Cl⁻) [adapted from (Evans et al., 2005)].

Species	Osmolarity, mOsm	Na ⁺ , mM	Cl ⁻ , mM	K ⁺ , mM	Ca ²⁺ , mM	Mg ²⁺ , mM
FW	1	0.25	0.23	0.005	0.07	0.04
Teleost	274	130	125	2.9	2.1	1.2

To mitigate solute loss and water loading, the gill, kidney, and gastrointestinal (GI) tract all work in concert to: (1) limit the outward flow of solutes across the gill epithelium; (2)

actively acquire solutes from the surrounding water across the gill epithelium as well as from diet; (3) recover solutes from the GI tract and through kidney filtration with the latter resulting in the production of relatively dilute and large volumes of urine (Fig. 3) (Evans, 2008). In this regard, our current understanding of how NO_2^- disrupts osmoregulation is limited to transcellular ion transport (Jensen, 2003), particularly across the gill epithelium, which is a major site of active ion acquisition in FW fishes. Since the gill epithelium interfaces directly with water, it must also deal directly with elevated waterborne levels of NO_2^- (Gaino et al., 1984; Williams and Eddy, 1986).

Hyposmotic FW Environment

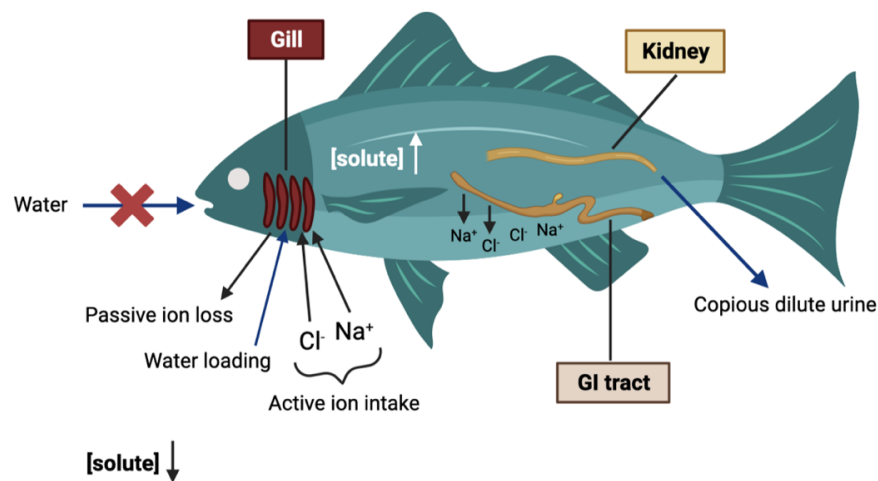


Figure 3: Freshwater (FW) teleost fish osmoregulation.

FW teleosts are hyperosmotic to their environment and must deal with water loading and passive ion loss. To maintain salt and water balance, FW teleosts rely on several strategies including: not drinking water; actively taking up ions across the gill; recovery of ions from the gastrointestinal (GI) tract via absorption of water from digestive contents, which also contains dissolved ions (primarily, sodium ions (Na^+) and chloride ions (Cl^-)), and kidney filtration; limiting the amount of passive ion loss; and producing voluminous dilute urine [adapted from (Evans et al., 2005; Evans, 2008); created with BioRender.com].

1.5 FW Teleost Gill Morphology

In fishes, the gills are found in the cephalic region, on either side of the pharynx (Fig. 4a). In teleosts, the gills are composed of four paired arch-like structures covered by a bony operculum that protects the branchial chamber. Each arch is formed by cartilaginous elements of the branchial skeleton, parts of which are calcified (Wilson and Laurent, 2002). From each arch, further extensions of the branchial skeleton, termed filaments, which are the basic functional unit of the gill, radiate in numerous rows (Fig. 4b). The filament supports rows of plate-like extensions, termed lamellae (Fig. 4c). The lamellae are particularly suited for gas exchange, given their large surface area, thin epithelial cell architecture, and vascular core: water enters the buccal cavity, flows over the filaments and lamellae countercurrent to the blood flow, and exits at the caudal opening of the operculum (Evans et al., 2005).

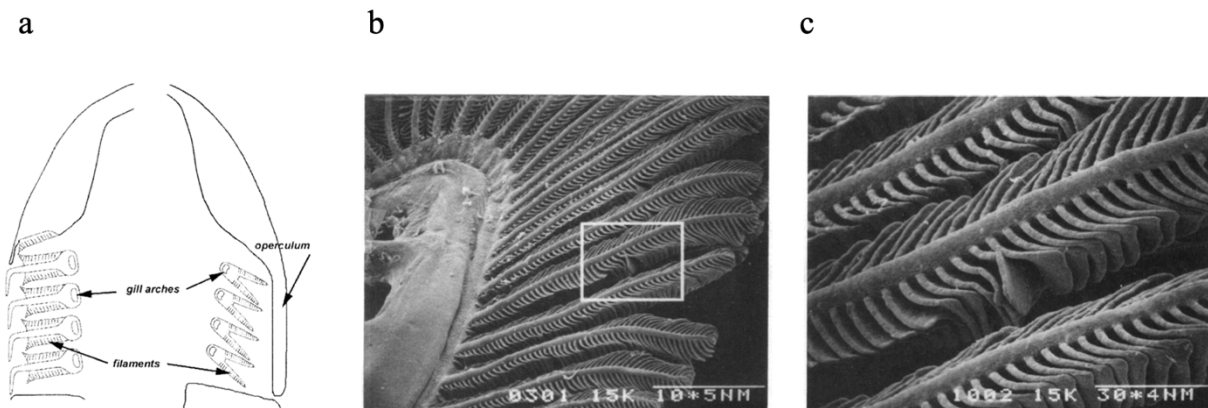


Figure 4: Freshwater (FW) teleost gill structure.

Panel (a) shows a schematic of the gill structure, including four paired gill arches and associated filaments, as well as the operculum, which covers the gills (Wilson and Laurent, 2002). Panel (b) shows a scanning electron micrograph of a branchial arch and gill filaments from the teleost gulf toadfish (*Opsanus beta*); the scale bar represents 10⁶ nm (1 mm) (Evans, 1987). Panel (c) shows a magnified view of the filaments and lamellae in the rectangular region of Panel (b); the scale bar represents 3 × 10⁵ nm (0.3 mm) (Evans, 1987).

The epithelium covering the filament and lamellae differs in terms of thickness, cell type, and underlying blood flow. The filament epithelium is typically composed of three or more cell layers. Most cells are cuboidal or squamous pavement cells (PVCs), while many mitochondria-rich cells (MRCs) are also present (Wilson and Laurent, 2002). In contrast, the lamella epithelium is thinner, typically less than three cell layers, and is primarily composed of PVCs.

The PVC is the most abundant cell type covering the gill epithelium (i.e., more than 90% of the surface area) (Wilson and Laurent, 2002), and its primary function is to form a relatively impermeable barrier between the surrounding water and tissue (Olson, 2011). In contrast to a PVC, which typically has a low number of mitochondria, an MRC has a high mitochondrial density (Fig. 5). The higher mitochondrial count is in line with the primary function of an MRC, which is ion transport, powered by ATP, for salt and water balance (Wilson and Laurent, 2002).

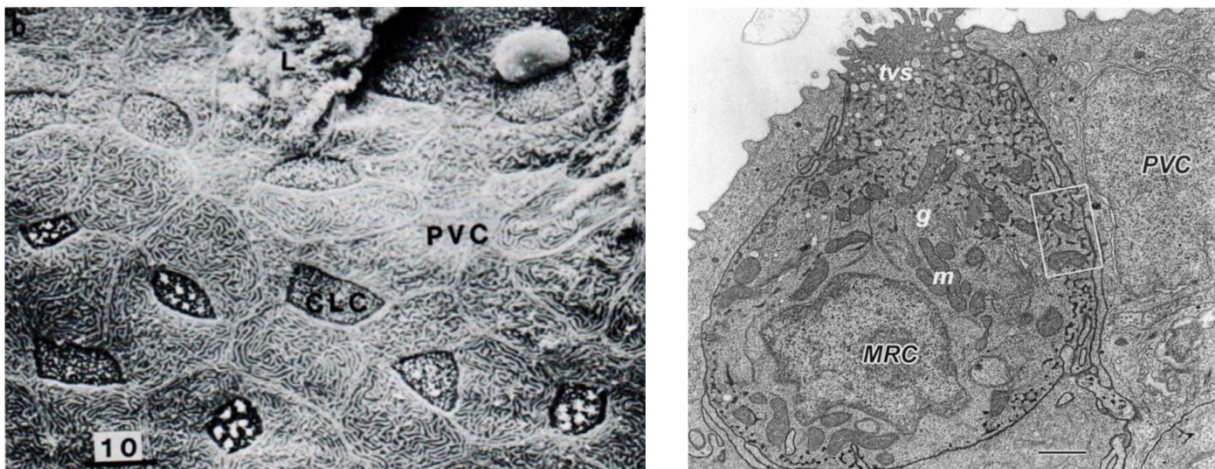


Figure 5: Electron micrographs of mitochondria-rich cells (MRCs) and pavement cells (PVCs) in a rainbow trout (*Oncorhynchus mykiss*) gill epithelium.

Micrograph of the trailing edge of a rainbow trout gill filament (left panel) showing MRCs (CLC) interspersed among PVCs. A PVC is shown next to an MRC (right panel). Scale bars represent 10 μm and 1 μm (left and right panels) (Laurent, 1984; Wilson and Laurent, 2002).

1.6 Transcellular Transport Across the FW Teleost Gill Epithelium

As noted above (Table 2), Na^+ and Cl^- are the dominant solutes in FW teleost blood plasma, and the transcellular transport of Na^+ and Cl^- across the gill epithelium has been widely studied. Presently, several models have been proposed for the transcellular Na^+ uptake in a FW teleost gill epithelial cell. In the most plausible model, a vacuolar type H^+ -ATPase (H^+ pump) actively pumps H^+ across the apical membrane into the surrounding water, creating an acidic environment for the uptake of Na^+ via an (H^+ -gated) acid-sensing ion channel (ASIC) (Fig. 6) (Lee et al., 2022). Here, intracellular generation of H^+ is through the hydration of carbon dioxide, which diffuses through the basolateral cell membrane, catalyzed by the enzyme carbonic anhydrase; this reaction also yields bicarbonate (HCO_3^-), which is instrumental for Cl^- uptake (Fig. 6).

Apical transcellular Cl^- uptake is generally assumed to be through a chloride-bicarbonate ($\text{Cl}^-/\text{HCO}_3^-$) anion exchanger (Perry et al., 2003). The basolateral exit of Na^+ is through a sodium-potassium ATPase (Na,K-ATPase) antiporter (Perry et al., 2003; Lee et al., 2022). Moreover, basolateral Cl^- exit is believed to be through a voltage gated chloride channel 2 (CLC-2) (Breves et al., 2017) (Fig. 6).

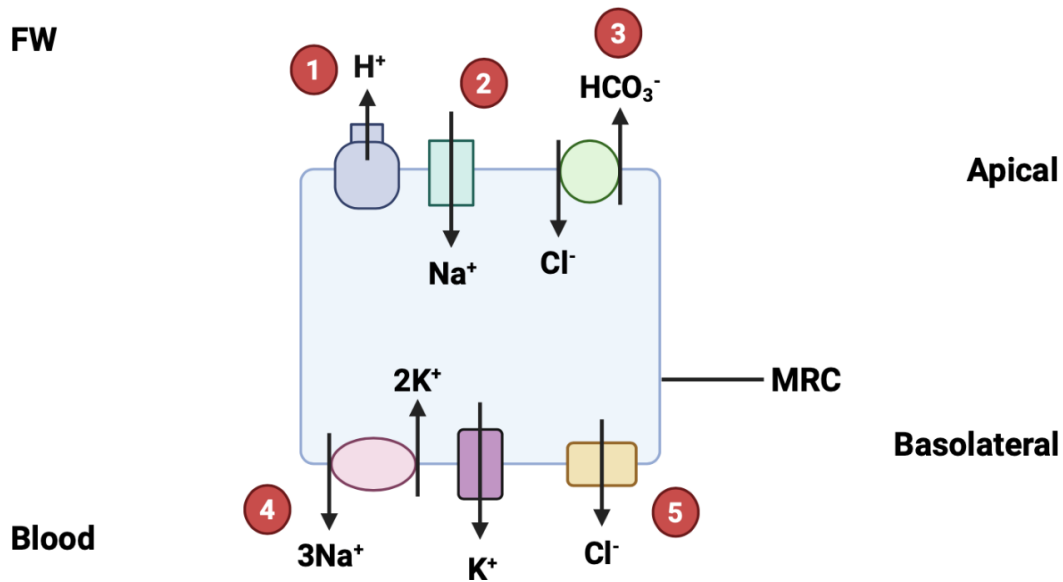


Figure 6: Schematic of freshwater (FW) teleost gill transcellular transport mechanisms across the gill mitochondria-rich cell (MRC).

Transcellular transport across a MRC utilizes: (1) a vacuolar type H^+ -ATPase, which actively pumps protons (H^+) across the apical membrane, (2) inward sodium ion (Na^+) uptake via an acid-sensing ion channel, (3) apical transcellular chloride ion (Cl^-) uptake through a chloride-bicarbonate ($\text{Cl}^-/\text{HCO}_3^-$) anion exchanger, (4) basolateral Na^+ exit via a sodium-potassium ATPase antiporter, (5) basolateral Cl^- exit believed to be via a chloride channel 2 [adapted from (Lee et al., 2022; Breves et al., 2017); created with BioRender.com].

At present, whether NO_2^- might impact the paracellular pathway of the gill epithelium - the principal route of passive ion efflux in the FW fish gill - has neither been suggested nor studied. However, there are reports showing that NO_2^- can alter the molecular components of the vertebrate epithelium tight junction (TJ) complex, which is the principal structural component controlling vertebrate epithelium permeability. For example, in a study by Rocha et al. (2016), Wistar rats were given dietary levels of NaNO_2 by oral gavage. Once in the stomach, NO_2^- was reduced to nitrogen monoxide (NO) by commensal bacteria. In turn, NO nitrated the tyrosine residues (thereby, converting tyrosine to nitrotyrosine) on the extracellular loops of occludin (Occludin; to be described below) protein in the TJs of epithelial cells next to the gastric lumen (Fig. 7). While the study did not address the functional impact of Occludin nitration in the gut, it did

speculate that this pathway may modulate gut barrier function with potentially negative implications for GI disorders such as inflammatory bowel disease (IBD).

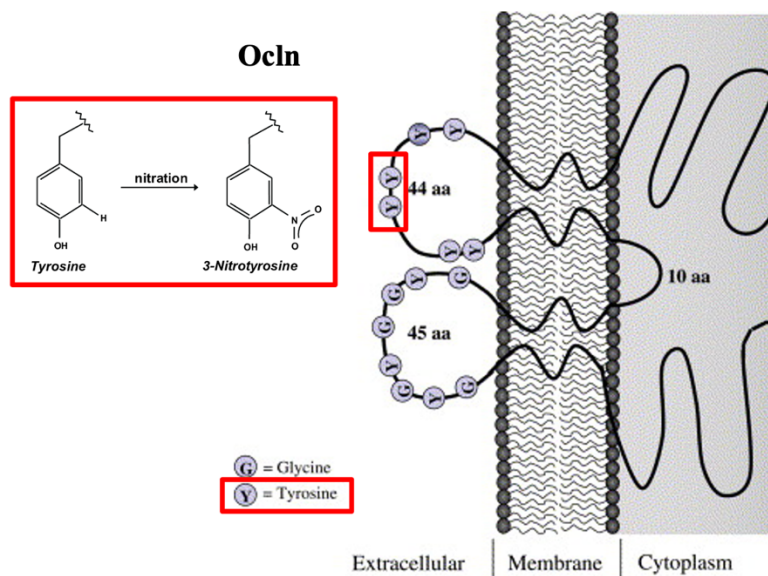


Figure 7: Nitrated occludin (Ocln).

Tyrosine residues on the extracellular loops of Occludin protein in the tight junctions (TJs) of gastric epithelial cells are converted by nitrogen monoxide to nitrotyrosine. Adapted from (Feldman et al., 2005).

1.7 Paracellular Transport Across the Vertebrate Epithelium

The TJ complex forms the most apical contact between adjacent cells in vertebrate epithelia and comprises multiple integral membrane and cytoplasmic proteins (Günzel and Yu, 2013). Cytoplasmic proteins anchor the TJ complex to the actin cytoskeleton. Integral membrane proteins control paracellular permeability by functioning as a gate, which allows selective transport of ion species dependent on size and/or charge, or as a barrier, which restricts paracellular diffusion (Günzel and Yu, 2013). Tetra-spanning integral proteins, which cross the plasma membrane four times, are the major determinants of paracellular permeability (Günzel and Fromm, 2012). These include the claudin (Cldn) class of proteins, of which more than 50

have been described in teleost fishes (Kolosov et al., 2020), as well as Ocln (Chasiotis et al., 2009).

Cldns have two extracellular loops, an intracellular loop, and a cytoplasmic tail (Fig. 8). The first extracellular loop forms a selectively permeable pore while the second extracellular loop participates in trans-interaction with Cldn proteins from the TJ complex of an adjacent cell (Günzel and Yu, 2013). The cytoplasmic tail appears to play a role in the exit of Cldns from the endoplasmic reticulum and trafficking to the TJ complex, as well as in binding to scaffolding proteins (Günzel and Yu, 2013). The scaffolding proteins include zonula occludens proteins 1-3 (ZO1-3), which bind directly to F-actin.

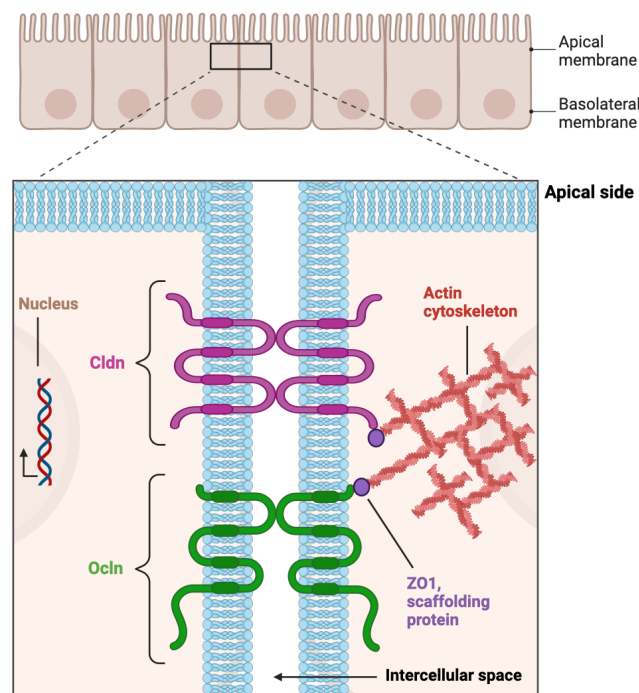


Figure 8: Schematic of the tight junction (TJ) complex in the freshwater (FW) teleost gill epithelium.

The TJ complexes of two adjacent FW teleost gill epithelium cells are depicted. Opposing tetraspanning claudin (Cldn) and occludin (Ocln) proteins are shown to interact near the apical side. The cytoplasmic tails of Cldn and Ocln proteins are shown to bind to the scaffolding protein zonula occludens 1 (ZO1), which attaches to the actin cytoskeleton [adapted from (Chasiotis et al., 2012a); created with BioRender.com].

The role of Cldns, as a barrier or gate, has been extensively studied. For example, Duffy et al. (2011) examined the role of Cldn-3a in the figure eight puffer fish (*Tetraodon biocellatus*) following acclimation from FW to ion-poor water (IPW), and observed an up-regulation of *cldn-3a* mRNA transcription (*cf.* Fig. 4a) while a study by Bagherie-Lachidan et al. (2008) on the green spotted puffer fish (*Tetraodon nigroviridis*) found a consistent result (*cf.* Fig. 6A), supporting the role of Cldn-3a as barrier forming.

Ocln is widely regarded to function as a barrier. For example, Chasiotis and Kelly (2011) noted a significant increase in *ocln* transcript levels in the gills of a common variety FW goldfish (*Carassius auratus*) following acclimation to IPW (*cf.* Fig. 4); moreover, Whitehead et al. (2011) showed that *ocln* mRNA levels in the gills of FW, mesohaline, and SW Atlantic killifish (*Fundulus heteroclitus*) were significantly increased following acclimation to IPW (*cf.* Fig. 3D).

In contrast to Cldns and Ocln (transmembrane TJ proteins that interact between two adjacent epithelial cells), transmembrane tricellular TJ proteins form at contact points of three neighbouring epithelial cells. Knockdown of the tricellular TJ protein tricellulin (TRIC) has been shown to significantly reduce transepithelial resistance and increase paracellular flux (Kolosov and Kelly, 2013), in the gill epithelium of rainbow trout (*O. mykiss*), supporting the function of TRIC as a barrier.

Presently, the impact of NO₂⁻ on salt and water balance has been studied for transcellular ion transport in the gill epithelium (Eddy and Williams, 1987; Jensen, 2003) while the potential impact on paracellular ion transport has been essentially unexplored.

1.8 Detrimental Effects on Salt and Water Balance in FW Teleost Fishes

NO₂⁻ sensitivity in fish varies widely. Table 3 shows NO₂⁻ lethal concentration 50 (LC50) values for various juvenile FW teleost fishes over approximately 96 hours; as can be seen, LC50

can differ by an order of magnitude depending on the species of fish. Given the detrimental impact of NO_2^- , the mechanism for NO_2^- uptake in the gill epithelium has been widely studied.

Table 3: Freshwater (FW) teleost nitrite (NO_2^-) toxicity.

Representative NO_2^- lethal concentration 50 (LC50) for various juvenile FW teleost fishes, determined over approximately 96 hours.

Organism	LC50, mg/l NO_2^-	Reference
Guppy (<i>Poecilia reticulata</i>)	30.2	Dolezelova et al., 2011
Rainbow trout (<i>Oncorhynchus mykiss</i>)	21.7	Kroupova et al., 2008
Zebrafish (<i>Danio rerio</i>)	242.6	Pistekova et al., 2005

Jensen (2003) noted substantial evidence for $\text{Cl}^-/\text{HCO}_3^-$ mediated transcellular NO_2^- uptake in the gill epithelium; in particular, saturation kinetics show that NO_2^- and Cl^- influx is carrier mediated while flux studies show that NO_2^- is a competitive inhibitor of Cl^- and vice versa (Williams and Eddy, 1986). Furthermore, Gaino et al. (1984) observed that NO_2^- is an inhibitor of carbonic anhydrase in the gill of rainbow trout (*O. mykiss*); however, its impact on branchial ion flow has not been further studied (Jensen, 2003).

As a result of NO_2^- exposure, part of Cl^- uptake is shifted to NO_2^- uptake while passive Cl^- loss persists, resulting in a net deficit of Cl^- (Jensen, 2003).

1.9 Hypothesis and Objective

Regarding transcellular processes, NO_2^- enters an MRC by outcompeting Cl^- in an apical $\text{Cl}^-/\text{HCO}_3^-$ anion exchange mechanism. Once inside the cell, NO_2^- inhibits the enzyme carbonic anhydrase (Gaino et al., 1984), resulting in a reduced yield of H^+ and HCO_3^- . Less H^+ availability in turn impacts transcellular Na^+ uptake via an ASIC, powered by an H^+ pump, which actively transports H^+ outward across the apical membrane creating an acidic external environment.

While this occurs, basolateral Na^+ exit through a Na,K-ATPase is disrupted due to lack of available Na^+ . Meanwhile, inhibition of carbonic anhydrase also impedes $\text{Cl}^-/\text{HCO}_3^-$ anion exchange, due to a reduced yield of HCO_3^- , and thus, inward NO_2^- uptake. The dynamics of transcellular NO_2^- uptake may then be governed by a negative feedback loop, resulting in cyclic levels of NO_2^- in the fish.

Is there any evidence to suggest that the detrimental NO_2^- effect on transcellular uptake processes may induce compensatory paracellular transport effects? The answer to this is simple but can also be nuanced. For example, there is no direct evidence, however, I would argue circumstances that result in a limited availability of ions (Na^+ and Cl^-) for a FW fish to acquire would be akin to the deleterious impact of elevated NO_2^- in water. In this regard, a study of Cl^- efflux in goldfish (*C. auratus*) that were acclimated to IPW showed a significant increase in the transcription of the barrier forming gene *ocln* and numerous barrier forming *cldns* (Fig. 9) (Chasiotis et al., 2012b). Here, IPW limits ion acquisition due to very low ion levels in the surrounding water. Therefore, if my study results in ion acquisition being limited due to competitive inhibition of transcellular Cl^- apical uptake, it could be considered reasonable to expect a similar outcome to Chasiotis et al. That is, in keeping with Chasiotis et al., who showed that as a compensatory response to reduced ion uptake, the TJs in the gill epithelium were tightened (thereby, reducing the rate of passive ion loss), it is reasonable to expect the same type of compensatory response for my study.

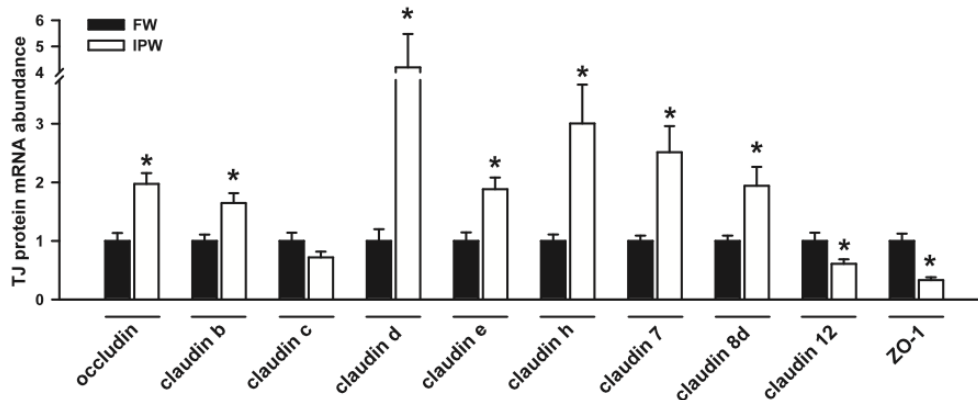


Figure 9: Gill tissue tight junction (TJ) mRNA relative abundance.

mRNA relative abundance after acclimation to ion poor water (IPW) increased for numerous barrier forming TJ proteins [reproduced from (Chasiotis et al., 2012b)]. ZO-1 denotes zonula occludens 1. * indicates significant variation from freshwater (FW) (p value < 0.05).

Moreover, there is evidence of direct NO_2^- impact on molecular components of the TJ in another vertebrate system. For example, Rocha et al. (2016) noted that nitration of the tyrosine residues on the extracellular loops of Ocln in the TJs of epithelial cells next to the gastric lumen of Wistar rats would lead to a leakier gut with potentially negative outcomes for GI disorders such as IBD (Section 1.6).

Since elevated waterborne NO_2^- impairs transcellular osmoregulatory mechanisms of the FW teleost gill epithelium and paracellular osmoregulatory mechanisms are part of the overall system for movement of solutes across the gill epithelium, I hypothesize that a compensatory response will result in an increase in the presence of barrier-forming TJ proteins, which would tighten the TJs of the FW teleost gill epithelium to counter the detrimental effects of NO_2^- on mechanisms of transcellular ion uptake. My objective is to consider this hypothesis by exposing guppy fish (*P. reticulata*) to varying levels of waterborne NO_2^- , after which I will examine the molecular physiology of the TJ complex. I will take an integrated approach to examine the molecular physiology of the guppy gill epithelium TJ complex. I will consider systemic

endpoints: (1) Na⁺ and Cl⁻ concentration in the whole-body carcass (organs and cephalic region removed) and (2) muscle moisture content. As well, I will consider molecular endpoints: (1) mRNA abundance of select TJ protein genes using qRT-PCR and (2) Na,K-ATPase activity. Furthermore, I will consider the morphology of the gill epithelium and gill epithelium TJ complex including the endpoint: qualitative ultrastructure analysis (gill epithelium cell types and TJ depth).

2 Materials and Methods

2.1 Experimental Design

2.1.1 Experimental Animals

To the best of my knowledge, no study has considered the effect of NO₂⁻ toxicity on male versus female fish, and as a practical first step, I have only used male fish in my study. Guppies were obtained from a local supplier, with an average weight of 0.32 ± 0.01 g and length 3.62 ± 0.02 cm (measurements are expressed as mean value plus/minus standard error of the mean (SEM)). Fish were separated into control (C), low (L), medium (M), high (H), and super high (SH) NO₂⁻ treatment groups (to be described below); moreover, to reduce the density and minimize the potential for spread of disease, each treatment group was subdivided into two subgroups and each subgroup was held in a separate 40 l aquarium. Fish were kept at room temperature (21-23 °C), under a 12/12-hour light/dark cycle, and were offered commercial guppy pellets once daily. Any uneaten food was removed after a 2-minute feeding period.

Before the start of experimentation, guppies were acclimated for 11 to 14 days (Table 4), depending on the treatment group; during the acclimation period, NO₂⁻ was *not* introduced to the aquaria. Following acclimation, NO₂⁻ concentration was gradually elevated to the level corresponding to the treatment group; this period is referred to as the NO₂⁻ graduation period. For

all aquaria, once NO_2^- concentration had reached its final level, fish were held in that condition for 7 days; this period is referred to as the NO_2^- experimentation period. For the SH group, for example, the acclimation period was 11 days, the graduation period was 4 days, and the NO_2^- experimentation period was 7 days, meaning that (1) fish were acclimated for 11 days (over which NO_2^- was not added to the aquaria), (2) NO_2^- was introduced over the 12th to 15th days, ratcheted up each day by one quarter of the full dose (i.e., 1/4, 2/4, 3/4, and full NO_2^- doses were applied on days 12, 13, 14, and 15, respectively), and (3) NO_2^- experimentation began on the 16th day and ended on the 22nd day. Moreover, the aquaria were equipped with an aquarium bubbler (for oxygenation) while filtration, which may have provided a substrate for bacterial growth, was omitted (Fig. 10).

Aquaria NO_2^- concentrations were maintained by adding NaNO_2 stock solution. The NaNO_2 stock solution was prepared using an appropriate mass of NaNO_2 crystalline powder (Thermo Scientific, MA, USA), dissolved in 200 ml of milli-q water, to yield a desired NO_2^- concentration. Water in treatment tanks was partially replaced (50% volume) every 48 hours with new, appropriately dosed FW (dechlorinated tap water). All water was monitored for NH_3 , NO_2^- , and NO_3^- using colorimetric assay kits.

Table 4: Fish acclimation and experimentation period status.

C, L, M, H, and SH denote control, low, medium, high, and super high nitrite (NO_2^-) treatment groups, respectively. Fish acclimation period (over which NO_2^- was not introduced to the aquaria) and NO_2^- graduation period (over which NO_2^- concentration was gradually ratcheted up to the final level), initial number and deaths over the combined acclimation and graduation periods as well as the NO_2^- experimentation period (7 days for all aquaria) are shown for all treatment groups. Deaths are comparable over all groups.

Treatment group	Tank no.	Acclimation period (days)	NO_2^- graduation period (days)	Fish status		
				Initial no.	Deaths	
					Acclimation and NO_2^- graduation	NO_2^- experimentation
C	1	13	0	18	0	2
	2			18	0	0
L	3	14	0	19	3	0
	4			18	0	0
M	5	12	2	18	1	3
	6			18	0	2
H	7	11	3	18	0	1
	8			18	0	2
SH	9	11	4	18	1	2
	10			18	0	0

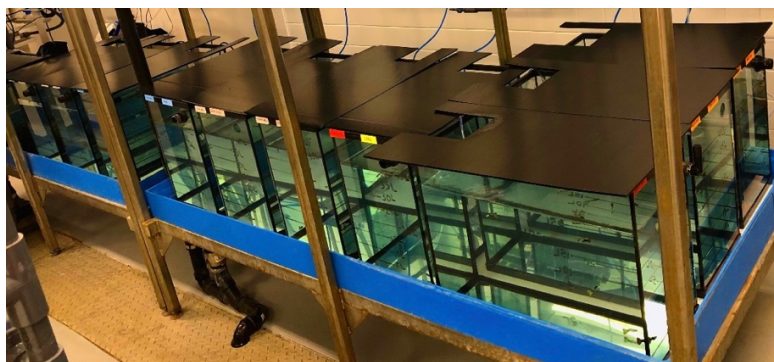


Figure 10: Nitrite (NO_2^-) exposure experimental set-up.

Ten 40 l aquaria, each holding $n = 18-19$ fish, were used for NO_2^- exposures.

2.1.2 NO₂⁻ Dose Selection

My study followed a dose-response, chronic exposure framework, whereby I exposed experimental fish to a series of sublethal NO₂⁻ doses for a 7-day period, before euthanizing the animals for tissue collection and analysis. Dosing was selected to be both environmentally and physiologically relevant.

For physiological relevance, I employed NO₂⁻ doses based on reduced proportions (Table 5) of the value for the guppy 96-hour LC50, 30 µg/ml, published in a study by Dolezelova et al. (2011). For environmental relevance, note that guppies are native to the FW systems of northern South America from western Venezuela to Guyana (de Mello et al., 2020). In Brazil, for example, guppies face increased degradation of water systems, driven by economic and urban growth. Brazil is one of the major consumers of agricultural fertilizer in the world, and agricultural fertilizer runoff is the greatest non-point source of NH₃ pollution of its FW systems (de Mello et al., 2020). In turn, excess NH₃ imbalances the N cycle, leading to increased levels of NO₂⁻ (Section 1.2). To the best of my knowledge, data regarding NO₂⁻ levels in Brazilian FW systems are not available. However, Eddy and Williams (1987) have surveyed NO₂⁻ levels in several other FW systems and have indicated that levels up to 2 µg/l N·NO₂⁻ occur in unpolluted surface FW while NO₂⁻ levels in polluted FW can be several orders of magnitude greater than unpolluted levels (Table 1). The largest NO₂⁻ dose in my experiment, 20 µg/ml for the SH group, is two thirds of the NO₂⁻ concentration in rivers receiving effluents from metal works (Table 1). Thus, my dose section is both physiologically and environmentally relevant.

Table 5: Experimental nitrite (NO₂⁻) dose selection.

NO₂⁻ doses employed for guppy chronic exposure. LC50 denotes lethal concentration 50; C, L, M, H, and SH denote control, low, medium, high, and super high NO₂⁻ treatment groups, respectively.

		Treatment group				
		C	L	M	H	SH
NO ₂ ⁻ dose	Percent of LC50 (30 µg/ml)	0	5	25	50	66
	Concentration, µg/ml	0	1.5	7.5	15	20

2.1.3 mRNA Analysis

Based on Chasiotis et al. (2012a), who reviewed the function of *cldn* and *ocln* genes in various fishes, and an mRNA expression profile of *cldn* genes in all organs of the guppy by Diakanastasis (2022), I examined several genes with the greatest mRNA abundance in the gills. These include the *ocln-a*, *b* and *cldn-3*, -8, -28, -30, and -31 orthologs, which are known to have a barrier forming role, as well as *cldn-10* orthologs, which are pore forming in SW teleosts but are present at a comparatively low level in MRCs of FW teleosts. As well, I examined *cldn-27a* and *TRIC*, which are likely barrier forming (Tipsmark et al., 2008; Kolosov and Kelly, 2013). To normalize abundance of these genes of interest (GOIs), I examined several housekeeping genes (HKGs), which were used only if they did not significantly alter in response to any of the experimental treatments (Tables 6 and 7).

Table 6: Tight junction (TJ) protein genes of interest (GOIs).

cldn, *ocln*, and *TRIC* denote *claudin*, *occludin*, and *tricellulin* genes, respectively.

GOIs	
Barrier forming TJ proteins	Mitochondria-rich cell associated
<i>cldn-3c, d</i>	<i>cldn-10a, b, c, d, e, f</i>
<i>cldn-8a, b, c, d</i>	
<i>cldn-27a</i>	
<i>cldn-28a, b, c</i>	
<i>cldn-30a, b, c, d</i>	
<i>cldn-31</i>	
<i>ocln-a, b</i>	
<i>TRIC</i>	

Table 7: Housekeeping genes (HKGs).

HKG	Short form
<i>ribosomal protein L13a</i>	<i>RPL13A</i>
<i>tubulin alpha-1B chain</i>	<i>TUBA1B</i>
<i>β-Actin</i>	<i>ACTB</i>
<i>18S ribosomal RNA</i>	<i>18S rRNA</i>

Studies have shown that HKG expression can vary widely depending on the cell line and experimental conditions. For example, a study by Hook et al. (2006), which examined patterns of gene expression in rainbow trout (*O. mykiss*) from sublethal exposure to endocrine disrupting chemicals, noted that several of the study toxicants caused changes in *ACTB* expression, and in those cases, qRT-PCR results were presented without *ACTB* normalization (an undesirable outcome). Moreover, Vandesompele et al. (2002) examined the relative expression levels of 10 commonly used HKGs in 13 different human tissues and found wide ranging tissue-specific differences in expression levels for certain genes (Fig. 11). They concluded that, to measure gene

expression levels accurately, normalization by multiple HKGs is required, and proposed the use of a normalization factor based on the geometric mean of at least three of the most stable HKGs. Following Vandesompele et al., I employed a relative expression ratio (Rel. Express. Ratio) (Pfaffl, 2001) of the form

$$Rel. Express. Ratio = \frac{E_{GOI}^{\Delta Ct_{GOI}}}{gm(E_{HKG_1}^{\Delta Ct_{HKG_1}}, \dots, E_{HKG_n}^{\Delta Ct_{HKG_n}})} \quad (1)$$

where Ct is the cycle threshold, $HKG_1, \dots, HKG_n$ denote n different HKGs, E_g is the efficiency of the gene g , and

$$gm(x_1, \dots, x_n) = \sqrt[n]{x_1 \cdot x_2 \cdots x_n}$$

is the geometric mean of the numbers $x_1, \dots, x_n$. The normalization factor in Equation (1),

$$gm(E_{HKG_1}^{\Delta Ct_{HKG_1}}, \dots, E_{HKG_n}^{\Delta Ct_{HKG_n}}),$$

was based on n of the best performing HKGs from Table 7, where the performance of a HKG was gauged by the consistency of its Ct values over all NO_2^- exposure treatment groups and samples within.

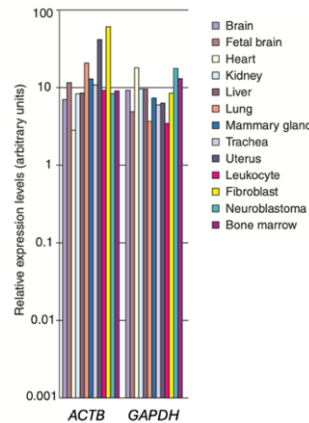


Figure 11: Housekeeping gene (HKG) expression profile in human tissues.

Normalized relative expression levels of HKGs vary according to tissue (*GAPDH* and *ACTB* denote *glyceraldehyde-3-phosphate dehydrogenase* and β -*Actin* genes, respectively). Adapted from Vandesompele et al. (2002).

2.2 Measurement of NO_2^-

NO_2^- was measured in experimental water to confirm that desired levels were reached and maintained. This was done colorimetrically using test solution for NO_2^- measurement acquired from an API water testing kit (API Freshwater Master Test Kit, NO_2^- test solution, USA). For NO_2^- standard curve determination, a series of dilutions with NO_2^- concentration in the range of 0 to $1.5 \mu\text{g/ml}$ was prepared using appropriate volumes of NO_2^- solution (NO_2^- ion chromatography standard solution, $1000 \mu\text{g/ml}$; Thermo Scientific, MA, USA) and milli-q water. Three drops of API NO_2^- test solution were then added to 3 ml of each dilution above. The absorbance of the resulting solutions was measured at 520 nm using a Thermo Scientific Multiskan Skyhigh spectrophotometer with 96-well microplate and SkanIT software (“spectrophotometer”; Life Technologies Holdings Pte Ltd, Singapore).

For aquarium NO_2^- concentration determination, a 5 ml sample of aquarium water was diluted with milli-q water, to produce an expected NO_2^- concentration of about $0.75 \mu\text{g/ml}$, to correspond with the approximate middle range of the NO_2^- standard curve.

2.3 NH_3 and NO_3^- Colorimetric Analysis

To measure the aquarium NH_3 and NO_3^- concentrations, a 5 ml sample of aquarium water was mixed with test solution volume/s according to manufacturer’s instructions (API Freshwater Master Test Kit, NH_3 and NO_3^- test solutions, USA) and the colour change was visually examined.

2.4 Tissue Sampling

Following Chasiotis and Kelly (2008), fish were net captured and anaesthetized in buffered tricaine methanesulfonate (MS-222, 0.5 g/l; Sydel Laboratories Ltd, Canada). Gill

tissue was removed, frozen in dry ice, and stored at -80 °C in TRIzol Reagent (Invitrogen, USA) for later RNA extraction and subsequent cDNA synthesis and qRT-PCR analysis. For Na,K-ATPase activity analysis, gill tissue was removed, frozen in dry ice, and stored at -80 °C for later use. For Na⁺ and Cl⁻ concentration analysis, the internal organs were removed, the cephalic region was cut away, and the remaining carcass was dried at 60 °C. For moisture content analysis, epaxial white muscle was dissected and dried at 60 °C. For transmission electron microscope (TEM) ultrastructure imaging, each gill arch was separated, placed in 2.5% glutaraldehyde in a phosphate buffer, and stored at 4 °C.

2.5 Primer Identification

Since a primer for the HKG *ACTB* had been previously developed by the Kelly Lab (Chasiotis and Diakanastasis, 2022a, b), primers for the HKGs *RPL13A*, *TUBA1B*, and *18S rRNA* (“the remaining HKGs”) had to be developed. Using the Nucleotide database, National Center for Biotechnology Information (NCBI), genes encoding the remaining HKGs, and their corresponding coding sequences (CDSs), were found for the Japanese puffer fish (*Takifugu rubripes*), which is closely related to the guppy and whose genome has been sequenced. The CDSs were submitted to the Primer3 Tool¹ for primer design, and the resulting primers were then submitted to the OligoAnalyzer Tool² to eliminate those with undesirable self-dimer and/or hairpin structures. The remaining primers were submitted to the Nucleotide BLAST³ tool to search for similar sequences in the guppy (BLASTn). Primers were optimized for annealing temperature, using the Thermo Scientific DreamTaq DNA Polymerase PCR protocol, and

¹ <https://primer3.ut.ee/>

² <https://www.idtdna.com/pages/tools/oligoanalyzer>

³ https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome

applied to cDNA from guppy whole-body and gill samples. Applying gel electrophoresis to the PCR product, amplicons of expected size were extracted from gels, using a QIAquick Gel Extraction Kit, and sent to The Centre for Applied Genomics (TCAG; SickKids) for DNA sequencing. The resulting DNA sequences were analyzed using the PubMed Nucleotide database, and those primers that did not match the HKG were re-run or redesigned. Primers for GOIs were developed, using the same procedure above, according to Chasiotis and Diakanastasis (2022a, b). See Tables 8 and A1 for HKG and GOI primer sequences, respectively.

Table 8: PCR primer sequences, amplicon length in base pairs (bp), and annealing temperature for housekeeping genes in the guppy (*Poecilia reticulata*).

ACTB, *RPL13A*, *TUBA1B*, and *18S rRNA* denote β -Actin, ribosomal protein L13a, tubulin alpha-1B chain, and 18S ribosomal RNA genes, respectively.

Gene	Primer sequence	Amplicon length, bp	Annealing temperature, °C
<i>ACTB</i>	FOR 5'-AGGGCGTGATGGTTGGTATG-3' REV 5'-CTGTTGGCTTTGGGGTTCAG-3'	226	60
<i>RPL13A</i>	FOR 5'-ATGAACACCAATCCGTCTCGTG-3' REV 5'-GGCAGCATTCCTCTAACAGTCCTC-3'	83	60
<i>TUBA1B</i>	FOR 5'-TCCCCAGAGCCTTTTTGTAGAC-3' REV 5'-GCAGCATCTTCCTTCCCAGTG-3'	115	60
<i>18S rRNA</i>	FOR 5'-CGATTCTGTGGGTGGTGGTG-3' REV 5'-AGTGGGGTTCAGCGGGTTAC-3'	283	60

2.6 mRNA Extraction, cDNA Synthesis, and qRT-PCR Analysis

Tissue sample total RNA extraction utilized TRIzol Reagent (Invitrogen, USA) and involved cell tissue lysis/homogenization, centrifugation, and RNA precipitation, following the manufacturer's instructions. Total RNA was then subjected to DNase I (Bio-Rad, Canada), to degrade DNA contaminants.

cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and a blend of oligo(dT) and random primers, according to the manufacturer's instructions (Bio-Rad iScript gDNA Clear cDNA Synthesis Kit, CA, USA).

qRT-PCR used gene-specific primers and the reaction conditions outlined in Kolosov et al. (2020). qPCR SSoAdvanced Universal SYBR Green Supermix (Bio-Rad, CA, USA) was used for signal amplification. A melting curve was generated to determine that amplification of non-specific primers or primer-dimers did not occur (Duffy et al., 2011).

Standard curves (logarithm of cDNA serial dilution concentration vs. Ct value) were constructed for each of the remaining HKGs and lines of best fit were used to determine efficiency values ($10^{-1/\text{slope of line}} - 1$), for use in Pfaffl gene relative expression analysis. An Excel spreadsheet was created to implement the Pfaffl method for gene relative expression (Pfaffl, 2001).

An expression profile of mRNA abundance, relative to that of a GOI with an average Ct value of 25-26 across all gill C samples, was produced.

2.7 Na,K-ATPase Activity

Gill Na,K-ATPase activity was measured using the McCormick (1993) method. Guppies were pooled into groups of two, and gill tissue was homogenized in a buffer of SEI and SEID (denoted by SEI:SEID, the buffer was a solution of SEI and SEID in a one to four volume ratio; SEI was a solution of 50 mM sucrose, 10 mM EDTA, 50 mM imidazole, and milli-q water at pH 7.3; and SEID was a solution of 13 mM sodium deoxycholate and SEI), in a 1 to 20 gill tissue weight to buffer volume ratio. The homogenate was centrifuged (6,900 rpm for 5 minutes at 4 °C) and the supernatant collected. ADP standards were prepared by mixing an aliquot of 4 mM ADP stock solution (57 nM ADP in a sodium acetate buffer, which was a solution of 2.8 mM

sodium acetate and milli-q water at pH 6.8) in an imidazole buffer (50 mM imidazole and milli-q water at pH 7.5).

Solution A (4 units/ml lactate dehydrogenase, 5 units/ml pyruvate kinase, 2.8 mM phosphoenolpyruvate, 3.5 mM ATP, 0.22 mM NADH, 50 mM imidazole, and milli-q water at pH 7.5), Salt Solution (189 mM sodium chloride, 10.5 mM magnesium chloride, 42 mM potassium chloride, 50 mM imidazole, and milli-q water at pH 7.5), and Working Solution A (Solution A and Salt Solution in a three to one volume ratio) were prepared.

ADP standards and Working Solution A were added to a 96-well plate. The plate was read using a spectrophotometer at 340 nm every minute until the slope of the ADP standard curve stabilized. Here, if the slope was less than 13, more NADH was added to Solution A, Working Solution A was prepared, and ADP standards were run again until the slope was over 13. The resulting slope was recorded for later calculations (see below).

Solution B (5 mM ouabain and Solution A) as well as Working Solutions A and B (Solution A and Solution B, respectively, and Salt Solution in a three to one volume ratio) were prepared for the number of samples run. Tissue samples were added to a 96-well plate, and appropriate volumes of Working Solutions A and B were added to half of the wells, respectively. The plate was read using a spectrophotometer at 340 nm every minute for 20 minutes, and NADH oxidation curves (NADH curves) were produced for those wells with Working Solutions A and B, respectively. Na,K-ATPase activity was calculated by

$$\frac{\frac{\text{Slope of NADH curve A} - \text{Slope of NADH curve B}}{\text{Slope of ADP standard curve}}}{\text{Protein concentration of sample}} \times 60,$$

expressed as μ moles ADP/mg protein/hour.

Protein concentration, expressed as $\mu\text{g}/10\ \mu\text{l}$ of sample, was determined by the Bradford (1976) assay. Gill tissue homogenate used for the Na,K-ATPase activity assay was diluted 4-fold with SEI:SEID buffer. Standard solutions were prepared by serially diluting Bovine Serum Albumin (BSA) stock solution (BSA diluted in milli-q water) in SEI:SEID buffer. Tissue homogenate samples and standards were pipetted onto a 96-well microplate. An appropriate volume of Bradford Reagent (Sigma-Aldrich, USA) was added to each well. The plate was shaken for 30 seconds, incubated for 5 minutes, and read using a spectrophotometer at 595 nm.

2.8 Muscle Moisture Content

White muscle moisture content was measured gravimetrically by drying the muscle to a constant weight at 60 °C and computing the relative difference between dry and wet muscle mass,

$$\frac{\text{wet weight} - \text{dry weight}}{\text{wet weight}} \times 100\%$$

(Chasiotis and Kelly, 2008).

2.9 Cl⁻ Concentration

Cl⁻ concentration was determined by colorimetric assay (Zall et al., 1956). Dry guppy whole-body carcass (organs and cephalic region removed) was digested in a volume of 1 M nitric acid (HNO₃) 20 times the weight of the dry carcass. The digest solution was centrifuged (13,000 rpm for 15 minutes at room temperature) and the supernatant collected. The supernatant was diluted 60-fold in 1% HNO₃. Then, 200 mM Cl⁻ stock solution (200 mM sodium chloride (NaCl) dissolved in 1% HNO₃) was serially diluted in 1% HNO₃ to prepare standards. Working Solution was prepared by mixing Solution A (148 mM mercuric thiocyanate dissolved in commercial grade methanol), Solution B (0.5 M ferric nitrate dissolved in a solution of 1 to 47

parts 68% HNO₃ and milli-q water), and milli-q water in a 1:1:13 ratio. Then, equal volumes of standard and sample were loaded on a 96-well plate and the same volume of Working Solution was added to each well. The plate was read using a spectrophotometer at 480 nm.

2.10 Na⁺ Concentration

Dry guppy carcass was digested and the supernatant collected as described in Section 2.9. Na⁺ concentration was measured by potentiometric analysis, using ion selective microelectrodes (ISMES), based on the protocol described in Lajevardi et al. (2021) as follows. Using a P-97 Flaming Brown pipette puller (Sutter Instruments Co., USA), unfilamented borosilicate glass capillaries were pulled with a tip diameter of ~5 μ m. The electrodes were then placed on a hot plate, baked for 15 minutes at 350 °C, silanized with dichlorodimethylsilane for one hour, and allowed to cool; here, silanization is necessary to coat the freshly pulled electrodes, which are hydrophilic, with a hydrophobic finish, allowing them to retain the hydrophobic ionophore (see below). To prepare reference electrodes, filamented borosilicated glass capillaries were pulled but not silanized.

To prepare Na⁺ selective ISMEs, the electrodes were backfilled with 100 mM NaCl and frontloaded with the ionophore solution Sodium ionophore II - cocktail A (Sigma-Aldrich, USA) to a column length of 200-250 μ m. To prevent leakage of the ionophore, the tips were coated in a polyvinyl chloride in tetrahydrofuran solution (Sigma-Aldrich, USA). Meanwhile, reference electrodes were backfilled with 500 mM potassium chloride. To calibrate the electrodes for ISME, voltages were read for two NaCl concentrations, a low concentration and a high concentration that was an order of magnitude larger than the low (0.5 and 5 mM, respectively), and the slope was computed, with an acceptable range between 50 and 62 mV. Since the carcass was digested in 1M HNO₃, to determine that the electrodes were not affected by the acid, the

aforementioned NaCl solutions were prepared by mixing an appropriate amount of NaCl with 1 M HNO₃ that was diluted with milli-q water by the same factor, 60 ×, as the sample supernatant. Sample and calibration droplets (5 μl) were placed into a petri dish with paraffin oil, and the LabChart 6 Pro software was used to record the ISME voltages. Na⁺ concentration was calculated as

$$[C]10^{\Delta V/m} \text{ mM}$$

where [C] is the concentration in mM of the NaCl solution used to calibrate the ISME, ΔV is the difference between the voltages of the sample supernatant and of the same calibration solution, and *m* is the slope.

2.11 TEM Imaging

The preparation of a sample for TEM imaging involves several steps. The first step, fixation, is based on Dykstra and Reuss (2003). Here, each gill arch was separated, fixed in 2.5% glutaraldehyde in a phosphate buffer (25% glutaraldehyde (Thermo Scientific, China), milli-q water, and phosphate buffer in a 1:4:5 volume ratio), and stored at 4 °C. The phosphate buffer was prepared by mixing Solutions A and B (0.2 M anhydrous disodium phosphate and 0.2 M sodium dihydrogen phosphate monohydrate, respectively, in milli-q water) in a 2.6 to 1 volume ratio. Further sample preparation - post-fixation, dehydration through a series of ethanol rinses, removal of residual ethanol by propylene oxide, and infiltration with Spurr resin - was completed by staff at the Nanoscale Biomedical Imaging Facility (NBIF), SickKids.⁴ Following infiltration, the samples were placed in embedding molds and polymerized in an oven at 65 °C overnight.

⁴ For the protocol, see https://lab.research.sickkids.ca/nbif/wp-content/uploads/sites/98/2020/11/SOP_Conventional-Tissue-preparation-for-TEM.pdf.

The samples were then sectioned (90 nm thick) on an ultramicrotome (Leica UC7) and collected on 200 mesh copper grids, stained with uranyl acetate and lead citrate.

2.12 Statistical Analysis

For all HKGs, each NO_2^- treatment group (C to SH), and all samples within each group, qPCR Ct values were run through the Grubb's test to determine outliers, and those that showed significant variation from the mean Ct value were removed. A Shapiro-Wilk test was run to determine whether the data is normally distributed. A parametric one-way ANOVA test, if the data is normally distributed, and otherwise, a non-parametric Kruskal-Wallis test, was then run to assess whether mean values were statistically different (p value < 0.05). Dunnett's or Dunn's post hoc analysis was used for multiple comparisons when ANOVA or Kruskal-Wallis indicated significant alteration, respectively. The computations above were done using the software Prism. The procedure above, beginning with the Grubb's test, was also carried out for all assays except for TEM.

3 Results

3.1 NO_2^- Standard Curve

Standard curves were employed to determine NO_2^- concentration in the aquaria. The standard curves showed a near perfect fit (Fig. 12).

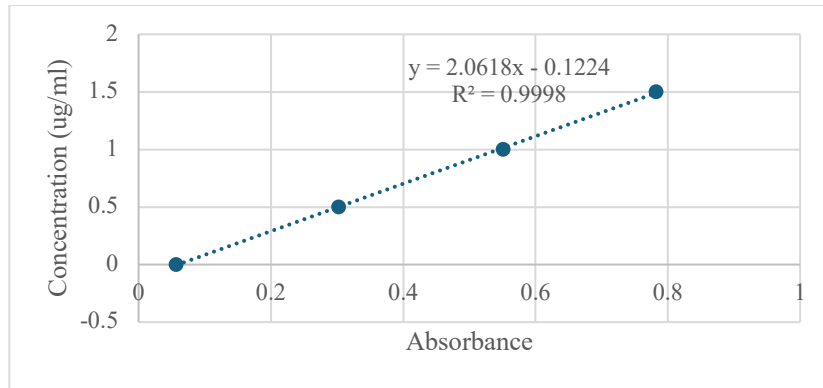


Figure 12: Representative standard curve produced from nitrite solutions with concentration ranging from 0-1.5 µg/ml.

Standard curve shows a near perfect fit ($R^2 = 0.9998$).

3.2 HKG Selection

I considered a basket of four HKGs and all combinations of three HKGs from this basket (Table 9). For a given basket, I used the Excel program Bestkeeper (Pfaffl et al., 2004) to (1) form a series of the geometric mean of the basket's constituent HKG Ct values over every treatment group and samples within, and (2) to compute the respective correlation between the series of geometric means in (1) and each series of the basket's HKG constituent Ct values over the same span. I chose the basket with the highest average correlation and least standard deviation (st. dev.), Basket 2 (*ACTB*, *RPL13A*, and *TUBA1B*; see Table 9).

Table 9: Basket of housekeeping genes (HKGs).

(a) composition of various baskets of HKGs under consideration (red highlight indicates the final selection of constituent HKGs); (b) correlation of basket constituent Ct values and geometric mean of Ct values (red highlight indicates the basket with highest mean correlation and lowest std. dev.). *ACTB*, *RPL13A*, *TUBA1B*, and *18S rRNA* denote β -Actin, ribosomal protein L13a, tubulin alpha-1B chain, and 18S ribosomal RNA genes, respectively.

(a)

Basket no.	HKGs			
	<i>ACTB</i>	<i>RPL13A</i>	<i>TUBA1B</i>	<i>18S rRNA</i>
1	✓	✓	✓	✓
2	✓	✓	✓	
3	✓	✓		✓
4		✓	✓	✓
5	✓		✓	✓

(b)

HKG	Basket				
	1	2	3	4	5
<i>ACTB</i>	0.83	0.94	0.78	-	0.84
<i>RPL13A</i>	0.88	0.8	0.89	0.89	-
<i>TUBA1B</i>	0.72	0.9	-	0.71	0.73
<i>18S rRNA</i>	0.92	-	0.97	0.96	0.92
Mean	0.84	0.88	0.88	0.85	0.83
St. dev.	0.086	0.073	0.094	0.128	0.095

3.3 Gill Gene Expression Profile

Using qRT-PCR, I generated a profile of gill gene transcript expression (Rel. Express. Ratio; Eq. (1)) for GOIs, normalized to the geometric mean of the transcript expression of Basket 2 constituent HKGs (“geometric mean of Basket 2”), relative to the transcript expression of *cldn*-

31 (average Ct value of 25.42; Fig. 13). Most genes were expressed with high mRNA abundance in the gills. However, *cldn-3d*, *-10d*, *-10e*, and *-30b* showed lower mRNA expression.

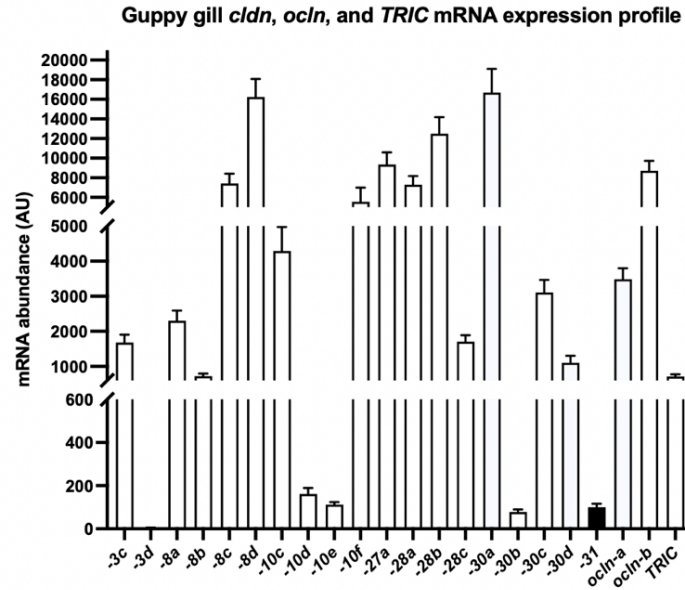


Figure 13: mRNA expression of various genes of interest (GOIs) in the gill.

cldn, *ocln*, and *TRIC* denote *claudin*, *occludin*, and *tricellulin* genes, respectively. All entries other than *ocln-a*, *-b* and *TRIC* correspond to *cldns*. GOI mRNA expression in the control group was normalized to the geometric mean of Basket 2 (*β-Actin*, *ribosomal protein L13a*, and *tubulin alpha-1B chain* housekeeping genes) and is expressed as a change relative to *cldn-31* mRNA abundance (assigned a value of 100; solid black bar). *cldn-10a*, *b* were quantifiable but not displayed ($Ct \approx 32$). Data are shown plus or minus standard error of the mean (SEM) ($n = 9$).

3.4 mRNA Abundance in the Gills

Figure 14 shows the mRNA abundance profiles for each GOI. *cldn-10e* mRNA expression was statistically lower than C for M to SH groups (Fig. 15). Except for *cldn-10e*, no other GOIs showed a statistical difference from C.

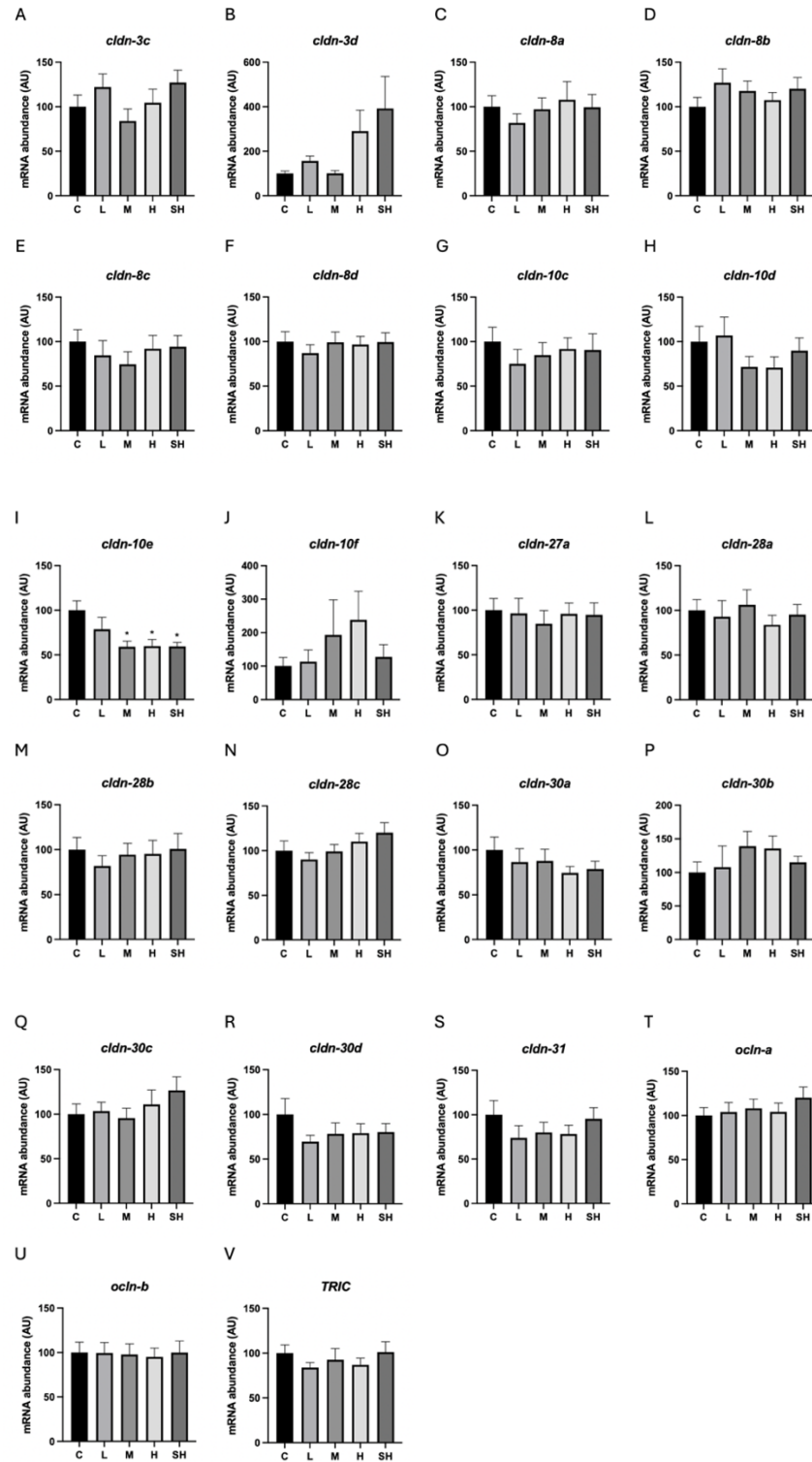


Figure 14: mRNA abundance of genes of interest (GOIs) in the gill.

Transcript expression of several GOIs normalized by the geometric mean of Basket 2 (*β -Actin*, *ribosomal protein L13a*, and *tubulin alpha-1B chain* housekeeping genes) was computed using

qRT-PCR and the Pfaffl Relative Expression Ratio $\frac{E_{GOI}^{\Delta Ct_{GOI}}}{gm(E_{HKG_1}^{\Delta Ct_{HKG_1}}, \dots, E_{HKG_n}^{\Delta Ct_{HKG_n}})}$, where Ct is the cycle threshold, $HKG_1, \dots, HKG_n$ denote n different HKGs, E_g is the efficiency of the gene g , and $gm(x_1, \dots, x_n) = \sqrt[n]{x_1 \cdot x_2 \cdots x_n}$ is the geometric mean of the numbers $x_1, \dots, x_n$. Data are expressed as mean value plus or minus SEM ($n = 8-10$). C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. *cldn*, *ocln*, and *TRIC* denote *claudin*, *occludin*, and *tricellulin* genes, respectively. C group mean value has been assigned 100 (black bar). * indicates significant variation from C (p value < 0.05).

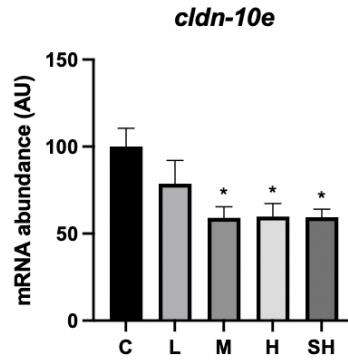


Figure 15: mRNA abundance of *claudin-10e* (*cldn-10e*) in the gill.

Transcript expression of *cldn-10e* extracted from Figure 14I. Data are expressed as mean value plus or minus SEM ($n = 8-10$). C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. C group mean value has been assigned 100 (black bar). * indicates significant variation from C (p value < 0.05).

3.5 Gill Na,K-ATPase Activity

For all treatment groups, sample Na,K-ATPase activity was found to be normally distributed, and therefore, a parametric one-way ANOVA was employed to test for statistical differences in Na,K-ATPase activity (see Table 10 for sample sizes). Na,K-ATPase activity trended downward for M to SH groups and was statistically lower than C for H and SH groups (Fig. 16).

Table 10: Treatment group size for sodium-potassium ATPase activity in the gill.

C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. Treatment group sample size varied from $n = 3$ to 6.

Treatment group	Sample size (n)
C, L, M	6
H	4
SH	3

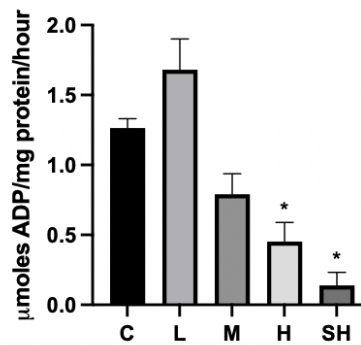


Figure 16: Gill sodium-potassium ATPase activity.

C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. Data are expressed as mean value plus or minus SEM. Data ($n = 3-6$) was normally distributed and showed statistical differences from C for H and SH groups. * indicates significant variation from C (p value < 0.05).

3.6 Muscle Moisture Content

Mean moisture contents of L to SH groups were not significantly different from C for white muscle (Fig. 17).

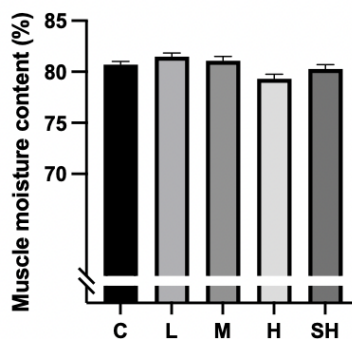


Figure 17: Muscle moisture content.

C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. Data are expressed as mean value plus or minus SEM. Moisture content data ($n = 11-12$) was normally distributed and did not show significant variation in the means.

3.7 Carcass Cl^- Concentration Endpoint

Carcass Cl^- concentration showed a downward trend. Only the Cl^- concentration of the SH group was statistically lower than C (Fig. 18).

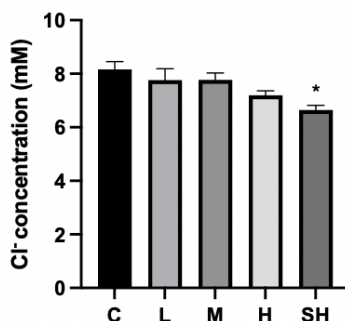


Figure 18: Carcass chloride ion (Cl^-) concentration.

C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. Carcass Cl^- concentrations are normally distributed, and Cl^- concentration for the SH group is statistically lower than C. Data are expressed as mean value plus or minus SEM ($n = 12$). * indicates significant variation from C (p value < 0.05).

3.8 Carcass Na^+ Concentration Endpoint

ISMEs were calibrated as described in Section 3.10 using low and high NaCl concentrations of 0.5 and 5 mM, respectively. The resulting slopes were within an acceptable

range for all treatment groups (Table 11). Carcass Na⁺ concentration increased slightly above the level for C, for the L group, but then followed a decreasing trend for M to SH groups (Fig. 19).

Only the concentration of the SH group was statistically lower than C (Fig. 19).

Table 11: Ion selective microelectrode calibration slopes.

C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. Slopes are all in the acceptable range of 50 to 62 mV. Data are shown as mean value plus or minus std. dev. (*n* equal to the no. of calibrations).

	Treatment group				
	C	L	M	H	SH
Av.	51.7	53.1	52.4	52.2	54.3
Std. dev.	0.0	1.2	0.9	1.8	2.9
No. of calibrations	1	3	4	2	6

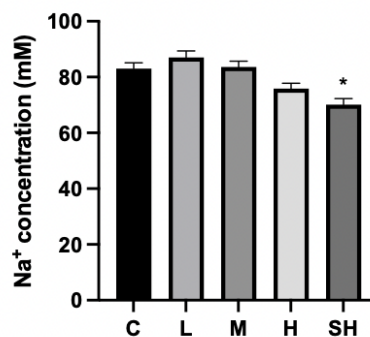


Figure 19: Carcass sodium ion (Na⁺) concentration.

C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. Carcass Na⁺ concentration for the SH group was statistically lower than C. Data are expressed as mean value plus or minus SEM (*n* = 12). * indicates significant variation from C (*p* value < 0.05).

3.9 Gill Epithelium Ultrastructure Imaging

TEM imaging was conducted using gills taken from C and SH treatment groups only. Imaging of a C gill (Fig. 20a) shows an MRC, bordered by PVCs and a blood vessel, with its apical membrane (AM) in direct contact with external FW. Within the MRC, various cellular components including tubules and mitochondria are highlighted. The red boxed area in Figure 20a (showing the apical border of an MRC and PVC) is magnified and shown in greater resolution in Fig 20b. A deep TJ adjoining the MRC and adjacent PVC can be seen (red square bracket, Fig. 20b). The MRC matrix shows numerous mitochondria, including mitochondrial cristae, as well as a system of tubules (Fig. 20c). The apical surface of the MRC in Figure 20a is comparatively flat and flush with the PVC surface, but another image of a C fish MRC (Fig. 20d) shows that an invaginated apical crypt (AC) could also occasionally be observed in these FW fish.

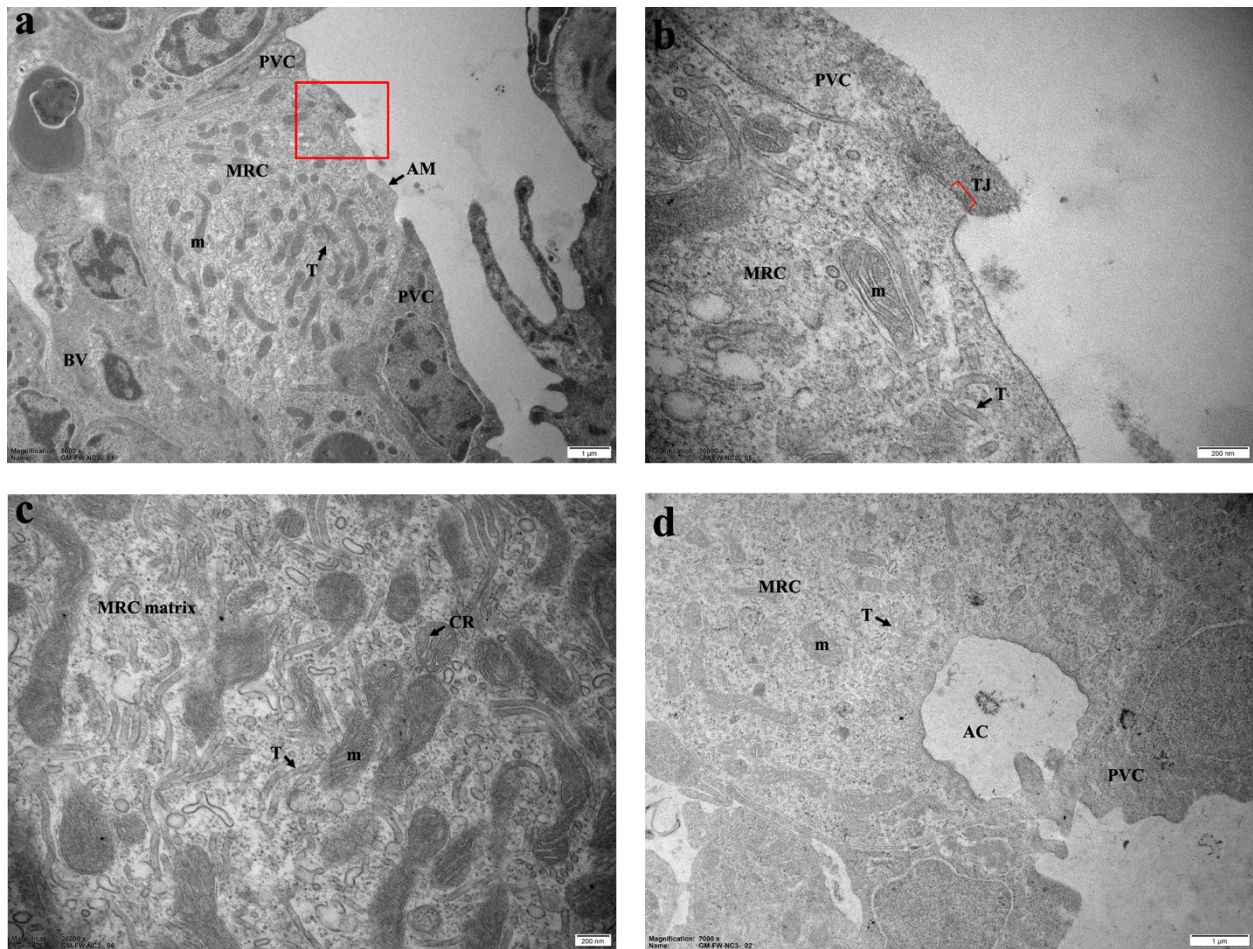


Figure 20: Control (C) treatment group gill epithelium transmission electron microscopy images.

Panel (a) shows a mitochondria-rich cell (MRC) bordered by pavement cells (PVCs) and with a blood vessel (BV) at its base. Its apical membrane (AM) is in direct contact with surrounding water and is comparatively flat and flush with the PVC surface. This was typically observed in C fish. Mitochondria (m) and tubules (T) can be easily observed in abundance within the matrix of the MRC. Panel (b), a magnification of the red boxed region in Panel (a), highlights the deep tight junction (TJ) between a bordering PVC and MRC (red square bracket). Panel (c) shows a magnified view of the MRC matrix, with numerous mitochondria, mitochondrial cristae (CR), and a system of tubules as indicated above. Panel (d) shows an MRC that presents an invaginated apical crypt (AC). In Panels (a-d), the scale bars represent 1 μm , 200 nm, 200 nm, and 1 μm , respectively.

Imaging of SH-exposed fish gill shows an MRC, bordered by PVCs, with its AM in direct contact with external FW (Fig. 21a). In contrast with C fish (Fig. 20a), mitochondria appear to be less dense and found further away from the AM. Boxed Region (i) (Fig. 21a) shows

an invaginated area of the MRC apical surface. A magnification of Boxed Region (ii) shows a deep TJ between a bordering PVC and MRC (Fig. 21b). The MRC matrix shows vacuolization in the tubular system (Fig. 21c). In SH-exposed fish gill, multiple MRCs sharing one apical opening could also be observed (Fig. 21d). A magnified view of the red boxed region in Figure 21d shows MRC-MRC TJs that appear to be shallow and/or loosely associated (red boxed region and red square bracket in Fig. 21e). MRCs with invaginated apical areas could also be more commonly observed in SH fish (Figs. 21d and f).

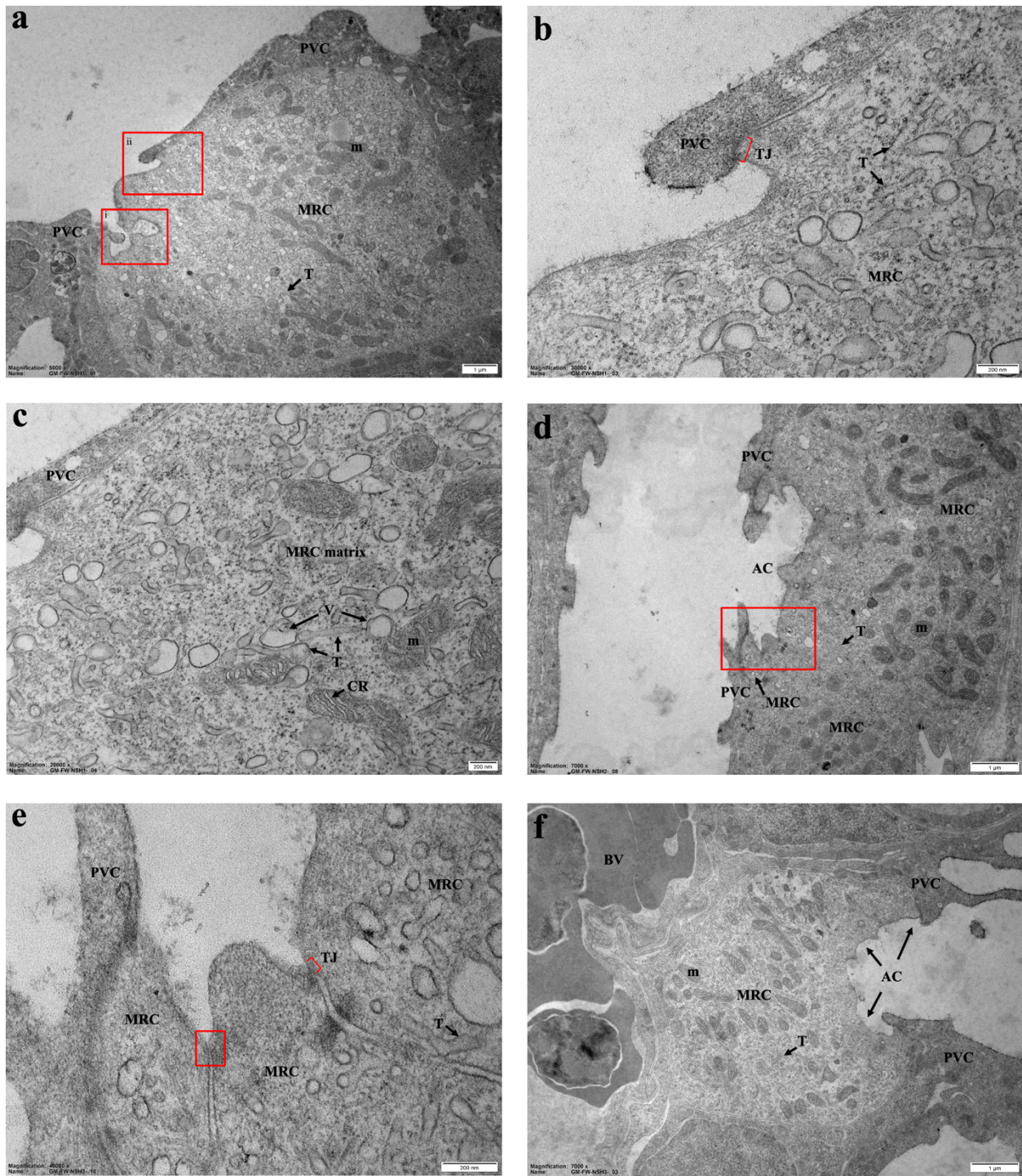


Figure 21: Super high (SH) nitrite treatment group gill epithelium transmission electron microscopy images.

Panel (a) shows a mitochondria-rich cell (MRC), flanked by pavement cells (PVCs), in direct contact with water. Boxed Region (i), in Panel (a), shows a deep invagination of the MRC apical surface with a wavy coastline while Boxed Region (ii) shows a shallower indentation of the apical surface. In Panel (a), mitochondria (m) and tubules (T) are highlighted, and are seen as

sparse and away from the apical membrane. Panel (b) shows a magnified view of Boxed Region (i), in Panel (a), and highlights a shallow tight junction (TJ) between a bordering PVC and MRC. Panel (c) shows the MRC matrix, highlighting mitochondrial cristae (CR) and vacuolization (V) in the tubular system. Panel (d) shows a central MRC, flanked by MRCs, with a deep invaginated apical crypt (AC). Panel (e) shows a magnified view of the red boxed region in Panel (d), highlighting a TJ with altered gill morphology (red boxed region) and a shallow TJ (red square bracket). Panel (f) shows an MRC that borders a blood vessel (BV) at its base. In Panels (a-f), the scale bars represent 1 μm , 200 nm, 200 nm, 1 μm , 200 nm, and 1 μm , respectively.

4 Discussion

4.1 Overview

I hypothesized that exposure of guppy fish to NO_2^- would result in a compensatory response in the gill epithelium resulting in an increase in the presence of barrier-forming TJ proteins. These barrier-forming TJ proteins would tighten the TJs of the NO_2^- -exposed FW guppy gill epithelium to counter the detrimental effects of NO_2^- on mechanisms of transcellular ion uptake. However, results were not as expected: only *cldn-10e* mRNA abundance was found to be statistically lower than C, suggesting a significantly lower count of MRCs compared to the C group (see Section 4.2.2). Nevertheless, Na,K-ATPase results showed a pronounced downward trend in activity for H and SH groups, indicating osmoregulatory impairment. Moisture content analysis was, unexpectedly, not statistically different from C but was consistent with similar studies in the literature. Carcass Na^+ and Cl^- concentration was statistically lower in the SH group, indicating significant impairment of ion uptake. TEM imaging of SH gill epithelium samples, in contrast to C samples, showed (1) a reduced number of mitochondria, which were located away from the apical membrane, (2) evidence of altered TJ morphology, and (3) vacuolization in the tubular system, indicating a compromised gill epithelium. Given these results, I cannot fully embrace my hypothesis.

4.2 Molecular Endpoints

4.2.1 Gill GOI Expression Profile

Greater mRNA abundance was noted for *ocln-a*, *b* as well as *cldn-3c*, *-8a-d*, *-10c*, *f*, *-27a*, *-28a-c*, *-30a*, *c*, *d* and *TRIC* genes (Fig. 13). These results are consistent with the following studies: Kolosov et al. (2014) produced a gene expression profile of rainbow trout (*O. mykiss*) in various organs and noted higher levels of *cldn-8d*, *-10c*, and *-28b* in the gill than in other organs. Bagherie-Lachidan et al. (2008) and Sun et al. (2015) reported strong expression of the *cldn-3c* ortholog in the gill of green spotted puffer fish (*T. nigroviridis*) and channel catfish (*Ictalurus punctatus*), respectively. Sun et al. also reported relatively high levels of *cldn-30a*, *c* expression in the gills of *I. punctatus*. Furthermore, Diakanastasis (2022) reported high expression of *cldn-8b-d*, *-10c*, *f*, *-28a-c*, *-30c*, *d* and moderate expression of *cldn-8a* in the gills of Endler's livebearer (*Poecilia wingei*).

4.2.2 Alterations in Gill TJ Protein mRNA Abundance in Response to NO₂⁻ Exposure

Compared to C, results showed statistically lower mRNA abundance in the gill only for *cldn-10e* (M to SH groups), which was unexpected. For greater context, Bui and Kelly (2014) examined *cldn-10e* expression in gill tissue of the euryhaline green spotted puffer fish (*T. nigroviridis*) under varying salinity conditions, and Cldn-10e protein was found to colocalize with Na,K-ATPases irrespective of salinity. Given that an abundance of Na,K-ATPase is a hallmark of MRCs, coupled with a previous study (Bui et al., 2010) that found *cldn-10e* to be absent from primary cultured *T. nigroviridis* PVCs, Bui and Kelly support that *cldn-10e* is abundant in, and may be specific to, MRCs in fishes. Then, a change in Cldn-10e protein abundance should reflect either a change in abundance within MRCs or if abundance remains the same, a change in the number of MRCs. Of course, a combination of these two variables could

also see changes in *Cldn-10e* abundance. With this in mind, there is evidence that NO_2^- exposure can alter MRC numbers in the gill epithelium of fish. For example, in a study by Williams and Eddy (1988), rainbow trout (*O. mykiss*) were exposed for 14 days to chronic levels of NO_2^- (4.6 mg/l NO_2^- , or about 21% of the rainbow trout 4-day LC50, 21.7 mg/l) comparable, as a proportion of LC50, to the M dose for my guppy experiment (25% of LC50, see Table 5), resulting in an approximately 50% increase in the number of MRCs per gram of gill tissue. Williams and Eddy viewed this increase as a compensatory response to counteract Cl^- depletion (since NO_2^- is a competitive inhibitor of Cl^- uptake). This view is consistent with Jensen (2003), who also noted, however, that such an increase may be detrimental, since it simultaneously elevates NO_2^- intake. While my qPCR results show a reduction of *cldn-10e* mRNA transcripts (M to SH groups; Figs. 14, 15), compensatory MRC proliferation may have resulted in a negative feedback loop, or apoptosis of MRCs, due to the increased NO_2^- influx. However, a more plausible explanation for the statistically lower *cldn-10e* mRNA abundance in M to SH groups is offered in Section 4.4, below, which discusses gill epithelium ultrastructure analysis.

4.2.3 Gill Na,K-ATPase Activity Analysis

Na,K-ATPase activity was statistically lower than C for elevated NO_2^- (H and SH groups). This result is consistent with a study by Woo and Chiu (1997) on the osmoregulatory effect of NO_2^- exposure in another species of teleost. In that study, sea bass (*Lates calcarifer*, a euryhaline teleost) in SW were exposed for four days to a series of sublethal NO_2^- concentrations (0, 30, 50, or 80 mg/l). Significantly decreased branchial Na,K-ATPase (an ion pump, which, in SW teleosts, is directly involved in salt secretion) activity compared to control (0 mg/l NO_2^-) was noted at 50 mg/l NO_2^- concentration, or 54% of the 96-hour LC50 (the effect of the highest NO_2^- concentration, 80 mg/l, on Na,K-ATPase activity was not reported). Given the sea bass' higher

tolerance for NO_2^- (in part, due to the higher Cl^- concentration in SW compared to FW), the reported sea bass statistically lower Na,K-ATPase activity at a high NO_2^- concentration supports my findings of significantly decreased Na,K-ATPase activity for guppy H and SH groups.

4.3 Systemic Endpoints of Salt of Water Balance

4.3.1 Muscle Moisture Content

Analysis did not show a significant difference in muscle moisture content for L to SH groups compared to C (Fig. 17). While these results are unexpected, they are consistent with a study by Woo and Chiu (1997), discussed in Section 4.2.3, that exposed sea bass (*L. calcarifer*) for four days to a series of sublethal NO_2^- concentrations, up to 86% of LC50, resulting in wide ranging osmoregulatory dysfunction (e.g., significantly decreased Na,K-ATPase activity) but no difference in muscle water content.

4.3.2 Carcass Cl^- Concentration

Carcass Cl^- concentration showed a downward trend in response to increasing levels of NO_2^- (Fig. 18), consistent with an expectation of increasing osmoregulatory impairment from exposure to elevated NO_2^- concentrations. However, only the SH group concentration was statistically lower than C, which may be explained by a study on the effect of NO_2^- exposure on another species of teleost. In a study by Jensen et al. (1987), carp (*C. carpio*) were exposed for 48 hours to 1 mM NO_2^- , and blood plasma Cl^- concentration was found to be statistically lower than control. Jensen et al. attributed the decrease in Cl^- concentration, in part, to competitive inhibition of $\text{Cl}^-/\text{HCO}_3^-$ exchange by NO_2^- . The NO_2^- concentration employed by Jensen et al. was about 9% of the carp 48-hour LC50, 510.504 mg/l (Eynizadeh et al., 2021), and approximately equivalent, as a percentage of LC50, to the L dose (5% of LC50) in my guppy study. Given that Jensen et al. considered Cl^- in the blood plasma, where it should be more

concentrated and sensitive to NO_2^- concentration compared to the carcass homogenate supernatant used in my study, their study lends support to the statistically lower Cl^- concentration observed in the SH group of my guppy study.

However, in a study by Gaino et al. (1984), rainbow trout (*O. mykiss*) exposed for 3 days to 1.5 mg/l NO_2^- , or about 7% of the rainbow trout 4-day LC_{50} , 21.7 mg/l (Dolezelova et al., 2011), did not show statistical difference from control in blood Cl^- concentration (the NO_2^- dose taken by Gaino et al. is comparable, as a proportion of LC_{50} , to the L dose for my guppy experiment). Moreover, ultrastructure analysis showed hypertrophy in MRCs treated with NO_2^- (an enhancement of total surface area leading to greater contact with the external environment and greater absorption of ions from the surrounding water). It may be that guppies follow a similar NO_2^- coping strategy, MRC hypertrophy, leading to lack of statistical difference in Cl^- concentration for L and M groups and a marginal level for the H group.

4.3.3 Carcass Na^+ Concentration

Analysis showed a downward trend in carcass Na^+ concentration as NO_2^- levels increased, and a statistically significant drop in concentration for the SH group compared to C (Fig. 19). These results are consistent with a study by Jensen et al. (1987) that measured Na^+ concentration in the blood plasma of carp (*C. carpio*) exposed for 48 hours to 1 mM NO_2^- . Plasma Na^+ concentration showed a downward trend and decreased to statistically lower values than control (onset of NO_2^- exposure) at 24 and 48 hours. Jensen et al. explain the decrease in concentration by the potential NO_2^- induced interference of Na,K-ATPase activity. Ciji and Akhtar (2020) further suggest the NO_2^- inhibition of carbonic anhydrase, leading to reduced production of H^+ and consequently, reduced influx of Na^+ as a factor.

4.4 Gill Epithelium Ultrastructure Analysis

TEM imaging of the gill epithelium showed MRCs with apical invaginations (AIs) in both C and SH groups (Figs. 20d and 21d). AIs were also reported by Pisam et al. (1987) in an ultrastructure study of guppy (*Lebistes reticulatus*) gill epithelium MRCs. Pisam et al. (1987) described two MRC types, α - and β -MRCs, respectively related to arterial and venous blood flow. Both MRC types displayed AIs while the β -MRC apical surface was noted to be generally more invaginated and wavy than that of the α -MRC. In a later study, Pisam et al. (1995) examined the gill epithelium MRC ultrastructure of FW tilapia (*Oreochromis niloticus*) adapted to IPW. The study identified α - and β -MRC types, and noted that in α -MRCs, upon transfer to IPW, apical folds were significantly increased while the apical cavity was usually deeper than FW. Since my study resulted in ion acquisition being limited due to competitive inhibition of transcellular Cl^- apical uptake (akin to limited ion acquisition due to very low ion levels in IPW), the deep and wavy invaginations found in the SH group (central MRC, see AC, Fig. 21a) are consistent with Pisam et al. (1987, 1995).

TEM imaging showed differences, between C and SH groups, in the MRC matrix composition as well as in the form of TJs. Compared to C (Fig. 20c), imaging of the MRC matrix in the SH group (Fig. 21c) showed fewer mitochondria, resulting in less ATP production and therefore, reduced Na,K-ATPase activity (consistent with my Na,K-ATPase activity results (Section 3.5)); and 2) vacuolization in the tubular system (Kikuchi, 1977), indicative of cellular damage or stress.

The finding of fewer mitochondria in my study may shed light on the statistically lower *cldn-10e* mRNA abundance noted in M to SH groups. Da Costa et al. (2017) examined changes to MRCs in the gill epithelium of tambaqui (*Colossoma macropomum*) exposed to NO_2^- . An

analysis of MRCs in fish exposed to NO_2^- noted 1) mitochondrial swelling, which has been related to the toxic action of NO_2^- in rat liver hepatocytes; 2) a statistically significant drop in the number of MRCs in the lamellar epithelium compared to control; and 3) a morphological pattern of necrosis (non-programmed cell death). Da Costa et al. attributed the statistically lower MRC count to necrosis, brought about by a sharp drop in ATP production after NO_2^- exposure. Since a change in Cldn-10e protein abundance should reflect a change in the number of MRCs (Section 4.2.2), MRC necrosis may be a plausible explanation for my *cldn-10e* mRNA abundance results. As further support for this explanation, note that TJs of the SH group were in some instances either shallow or at least lacked typical deep morphology (Figs. 21b and e), in contrast to the regular TJs in C (Fig. 20b), indicating osmoregulatory system damage (since FW teleost are hyperosmotic to their external environment and would seek to minimize passive ion loss through deep TJs (Section 1.4)).

Finally, observe that the proximity of an MRC to a blood vessel (Fig. 21f) underpins the organism wide distribution of NO_2^- (with its associated deleterious effects).

5 Conclusion and Future Work

The present study examined the molecular physiology of the guppy (*P. reticulata*) gill epithelium TJ complex after exposure to sublethal levels of waterborne NO_2^- . I hypothesized that a compensatory response, to counter the detrimental effects of NO_2^- on mechanisms of transcellular ion uptake, would increase the presence of barrier-forming TJ proteins. However, molecular endpoint results did not support this hypothesis: mRNA abundance did not significantly alter from C, for any of the TJ genes and all NO_2^- treatment groups, except for *cldn-10e*, which was statistically lower than C for elevated levels of NO_2^- (M to SH groups). Moreover, TEM imaging of an MRC exposed to an elevated level of NO_2^- (SH) showed fewer

mitochondria than C, and Na,K-ATPase activity (H and SH groups) was statistically lower than C while carcass Na^+ and Cl^- concentrations, which were found to significantly reduce in response to elevated NO_2^- levels, were consistent with osmoregulatory imbalance. Taken together, the TEM imaging and Na,K-ATPase activity results suggest low levels of ATP production for MRCs exposed to elevated NO_2^- . Low levels of ATP were assumed to be the likely driver of MRC necrosis in a recent study that exposed tambaqui (*C. macropomum*) to elevated levels of NO_2^- . Since a change in Cldn-10e protein abundance should reflect a change in the number of MRCs, MRC necrosis might also explain my finding of statistically lower *cldn-10e* mRNA abundance. As well, it may shed light on why there was no statistical decrease in mRNA abundance of non-MRC associated GOIs tested, since MRCs make up less than 10% of the gill epithelium. Thus, as my study did not show evidence of tightening of TJs after exposure to waterborne NO_2^- - on the contrary, TEM imaging of the SH group showed instances of shallow TJs or TJs with altered gill morphology - I reject my hypothesis.

Future work should focus on gill epithelium MRCs. It could include a numerical count of MRCs, ultrastructure morphometrics of mitochondria in MRCs to determine their health after exposure to NO_2^- , and a study of mitochondrial pathways that are affected by NO_2^- . As well, it could employ a primary culture of MRCs to conduct further molecular or cytology studies. While my study did not end as expected, it did identify a potential strategy taken by the guppy to conserve energy after NO_2^- exposure: MRC necrosis. Given the worldwide increased anthropogenic input of NO_2^- into FW systems, the study of toxicological effects of NO_2^- on fishes and compensatory strategies becomes even more pressing.

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Appendix A: Genes of Interest Primers

Table A1: PCR primer sequences, amplicon length in base pairs (bp), and annealing temperature for genes encoding *claudins* (*cldns*), *occludin* (*ocln*), and *tricellulin* (*TRIC*) protein genes in *Poecilia reticulata*.

Primers were created and confirmed by Chasiotis and Diakanastasis (2022a, b).

Gene	Primer sequence	Amplicon length, bp	Annealing temperature, °C
<i>cldn-3c</i>	FOR 5'-GATGGGTTTGGAGATTGTGG-3' REV 5'-TGCGAAGTGATGATGTTGG-3'	123	55
<i>cldn-3d</i>	FOR 5'-CAGATTGTGCCCCGTTTCCTG-3' REV 5'-TTAGTGCCTTCTCCTCCTGTGG-3'	187	60
<i>cldn-8a</i>	FOR 5'-GAACTGTGGGAAGGTCTGTG-3' REV 5'-GGCTGGGAGAATCAATAATG-3'	93	55
<i>cldn-8b</i>	FOR 5'-GTGTTGCTCTCTGGCTCTGG-3' REV 5'-CACAGGAATAAGGTCACAAATGC-3'	160	60
<i>cldn-8c</i>	FOR 5'-ACCCGTGTCTTGGACTGGTC-3' REV 5'-GGATGCTTGGAATGCTGGAG-3'	275	60
<i>cldn-8d</i>	FOR 5'-CAAGCCACCAATGTCCTATGC-3' REV 5'-TCCCAGCGAGTTTCCATCAC-3'	285	60
<i>cldn-10a</i>	FOR 5'-TGTGGAAAATGTGCGTGACTG-3' REV 5'-AGACGAGCCTTGGTGGTGTC-3'	202	60
<i>cldn-10b</i>	FOR 5'-TGCTCACTTTCTGCCTGTTCC-3' REV 5'-GCTTCGTCTGCCCTCTTGTG-3'	262	60
<i>cldn-10c</i>	FOR 5'-GGGTGGATTTTGGTCTGTTCC-3' REV 5'-TGATGGAGGTGATGATGAGAGC-3'	310	60
<i>cldn-10d</i>	FOR 5'-GGTGCTGCTGTTTTTCATTGG-3' REV 5'-CATACGAAGGTGTCCCGATTC-3'	281	60
<i>cldn-10e</i>	FOR 5'-TAGGTGGAATGGGTGGTTCG-3' REV 5'-GCAGGGAAATCATAGCAGTTGG-3'	112	60
<i>cldn-10f</i>	FOR 5'-CATTTCTGGGTTTGGTGGGATG-3' REV 5'-CATTGAGTCGGGAGGGTTGG-3'	186	60
<i>cldn-27a</i>	FOR 5'-CGTCGTCTCCGTCTTGTTG-3' REV 5'-ATCGCTCGTCCTCAATGC-3'	86	55
<i>cldn-28a</i>	FOR 5'-CTGGGGTTCTGGTGTGTTGGTC-3' REV 5'-TAGATTTGGCGACGGATGTG-3'	264	60
<i>cldn-28b</i>	FOR 5'-GATGCTCAGAAGAGAGAGTTGG-3' REV 5'-TATTGCTGCTTGTGTTGGTGTC-3'	170	55

Table A1 (continued)

Gene	Primer sequence	Amplicon length, bp	Annealing temperature, °C
<i>cldn-28c</i>	FOR 5'-CAGGTGTTTTGGGAAGGTC-3' REV 5'-GTCGTAGCGGCAGAAGTTG-3'	204	55
<i>cldn-30a</i>	FOR 5'-CATCGCTCTTCATCGGTTG-3' REV 5'-GTCTTTGGGAGGGCAGTTG-3'	83	55
<i>cldn-30b</i>	FOR 5'-GGAGAAGGCTAAGGCGAAGG-3' REV 5'-AAACGGGAACGAGGCAGAG-3'	383	55
<i>cldn-30c</i>	FOR 5'-AAAGGTCAAGGTGGGCATTG-3' REV 5'-AAAGTTCTGGATGATGGTGTTGG-3'	103	60
<i>cldn-30d</i>	FOR 5'-TCAGTTCGCTATGTGGTAGTCC-3' REV 5'-ATGGGTTTGATGGGTTGAG-3'	101	55
<i>cldn-31</i>	FOR 5'-CCTTTACCTCGTTCCTGTCTGC-3' REV 5'-GGGACTGCTTTCTCCACTGC-3'	220	60
<i>ocln-a</i>	FOR 5'-ACCCCTCATCCCAGACATC-3' REV 5'-ACCAAGAAGCCGAACACAAC-3'	163	55
<i>ocln-b</i>	FOR 5'-GCACAATACCAGACACAGACTCAGG-3' REV 5'-TTCACCCGACACATTCTTCACC-3'	267	60
<i>TRIC</i>	FOR 5'-CAGAACCCCAGCACCCAAAC-3' REV 5'-TCAAATACTCGCAACGCTCCTTC-3'	115	60

Appendix B: *p* values

Table B1: *p* values for gill genes of interest (GOIs) mRNA abundance.

cldn, *ocln*, and *TRIC* denote *claudin*, *occludin*, and *tricellulin* genes, respectively. C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. Sample values for each treatment group are indicated to be normally distributed or not. If normal, Dunnett's, and if not normal, Dunn's multiple comparison tests were employed to identify statistically significant differences from C (*p* value < 0.05; highlighted in green). For those groups whose sample values were not normally distributed, they were log-transformed but did not alter in statistical significance and hence, the transformed results are omitted. *cldn-10e* mRNA abundance for groups M-SH showed a statistically significant difference from C.

GOI	Treatment group	Normal (Y/N)	<i>p</i> value	
			Dunnett	Dunn
<i>cldn-3c</i>	L	N		>0.9999
	M			>0.9999
	H			>0.9999
	SH			0.6463
<i>cldn-3d</i>	L	N		0.5268
	M			>0.9999
	H			0.1433
	SH			0.0586
<i>cldn-8a</i>	L	N		0.7133
	M			>0.9999
	H			>0.9999
	SH			>0.9999
<i>cldn-8b</i>	L	Y	0.3237	
	M		0.699	
	H		0.9737	
	SH		0.5729	
<i>cldn-8c</i>	L	N		>0.9999
	M			0.4525
	H			>0.9999
	SH			>0.9999
<i>cldn-8d</i>	L	Y	0.788	
	M		>0.9999	
	H		0.9978	
	SH		>0.9999	

Table B1 (continued)

GOI	Treatment group	Normal (Y/N)	<i>p</i> value	
			Dunnett	Dunn
<i>cldn-10c</i>	L	N		0.3805
	M			>0.9999
	H			>0.9999
	SH			>0.9999
<i>cldn-10d</i>	L	Y	0.9937	
	M		0.5428	
	H		0.4686	
	SH		0.9728	
<i>cldn-10e</i>	L	N		0.2479
	M			0.0178
	H			0.0129
	SH			0.0316
<i>cldn-10f</i>	L	N		>0.9999
	M			>0.9999
	H			>0.9999
	SH			>0.9999
<i>cldn-27a</i>	L	N		>0.9999
	M			0.9702
	H			>0.9999
	SH			>0.9999
<i>cldn-28a</i>	L	N		>0.9999
	M			>0.9999
	H			>0.9999
	SH			>0.9999
<i>cldn-28b</i>	L	N		>0.9999
	M			>0.9999
	H			>0.9999
	SH			>0.9999
<i>cldn-28c</i>	L	Y	0.873	
	M		>0.9999	
	H		0.8572	
	SH		0.382	

Table B1 (continued)

GOI	Treatment group	Normal (Y/N)	<i>p</i> value	
			Dunnett	Dunn
<i>cldn-30a</i>	L	N		>0.9999
	M			>0.9999
	H			0.6738
	SH			>0.9999
<i>cldn-30b</i>	L	N		>0.9999
	M			0.3838
	H			0.3275
	SH			>0.9999
<i>cldn-30c</i>	L	N		>0.9999
	M			>0.9999
	H			>0.9999
	SH			0.6044
<i>cldn-30d</i>	L	Y	0.2254	
	M		0.5381	
	H		0.5207	
	SH		0.5946	
<i>cldn-31</i>	L	N		0.4251
	M			>0.9999
	H			>0.9999
	SH			>0.9999
<i>ocln-a</i>	L	Y	>0.9999	
	M		>0.9999	
	H		>0.9999	
	SH		0.9737	
<i>ocln-b</i>	L	N		>0.9999
	M			>0.9999
	H			>0.9999
	SH			>0.9999
<i>TRIC</i>	L	N		0.7367
	M			>0.9999
	H			0.8612
	SH			>0.9999

Table B2: *p* values for gill sodium-potassium ATPase activity.

C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. Sample values for each treatment group were normally distributed. Dunnett's multiple comparison tests were employed to identify statistically significant differences from C (p value < 0.05). H and SH groups showed a statistically significant difference from C.

Treatment group	<i>p</i> value
L	0.1687
M	0.0995
H	0.0069
SH	0.0008

Table B3: *p* values for muscle moisture content.

C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. Sample values for each treatment group were normally distributed. Dunnett's multiple comparison tests were employed to identify statistically significant differences from C (p value < 0.05). Analysis showed no statistically significant differences.

Treatment group	<i>p</i> value
L	0.4693
M	0.8972
H	0.061
SH	0.8686

Table B4: *p* values for carcass chloride ion concentration.

C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. Sample values for each treatment group were normally distributed. Dunnett's multiple comparison tests were employed to identify statistically significant differences from C (p value < 0.05 ; highlighted in green). SH group showed a statistically significant difference from C.

Treatment group	<i>p</i> value
L	0.7058
M	0.7135
H	0.0557
SH	0.0012

Table B5: p values for carcass sodium ion concentration.

C, L, M, H, and SH denote control, low, medium, high, and super high nitrite treatment groups, respectively. Sample values for each treatment group were not normally distributed. Dunn's multiple comparison tests were employed to identify statistically significant differences from C (p value < 0.05 ; highlighted in green). SH group showed a statistically significant difference from C.

Treatment group	p value
L	>0.9999
M	>0.9999
H	0.1298
SH	0.0038