Effect of temperature and sex on the molecular physiology of the Poeciliid gill epithelium tight junction complex

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ABSTRACT

Temperature influences ionoregulation in fishes, yet its effects on the gill epithelium tight junction (TJ) complex and sex-specific responses remain poorly understood. This study examined the gill epithelium TJ complex of male and female *Poecilia reticulata* following acclimation to high (33°C), medium (25°C, control), and low (17°C) temperatures. No sex-specific effects were observed for carcass moisture and Cl^- content. When sexes were pooled, carcass Cl^- decreased in both temperature extremes. Gill Na^+/K^+-ATPase activity decreased in males at low temperature. At high temperature, transcript abundance of heat shock protein 70 (*hsp70*) and *hsp90* significantly increased in male and female fish, confirming cellular stress. Temperature stress altered the transcript abundance of genes encoding TJ-associated proteins, particularly barrier-forming claudin (Cldn), *cldn3, cldn8, cldn30,* and *cldn31*. Females had greater *cldn10b* and *cldn10d* transcript abundance. These findings suggest that selective, sex-specific regulation of gill paracellular and transcellular ionoregulatory mechanisms occur in response to temperature stress.

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LIST OF ABBREVIATIONS

ANOVA – Analysis of variance

ASIC – Acid-sensing ion channel

cDNA – Complementary DNA

Cl^- – Chloride ion

CLDN – Claudin (mammalian protein)

Cldn – Claudin (fish protein)

cldn – Claudin gene

Ct – Cycle threshold

CTmax – Critical thermal maximum

CTmin – Critical thermal minimum

FW – Freshwater

GI – Gastrointestinal

HSP – Heat shock protein (mammalian protein)

Hsp – Heat shock protein (fish protein)

hsp – Heat shock protein gene

MDCK – Madin-Darby canine kidney

MR – Mineralocorticoid receptor protein

mr – Mineralocorticoid receptor gene

MRC – Mitochondria-rich cell

mRNA – Messenger RNA

Na^+ – Sodium ion

NKA – Na^+/K^+-ATPase

OCLN – Occludin (mammalian protein)

Ocln – Occludin (fish protein)

ocln – Occludin gene

PVC – Pavement cell

qRT-PCR – Quantitative real time polymerase chain reaction

rRNA – ribosomal RNA

TJ – Tight junction

ZO-1 – Zonula occludens-1 protein

zo-1 – Zonula occludens-1 gene

1. INTRODUCTION

1.1 Overview

The ability of fishes to precisely regulate salt and water balance is essential for their survival (Evans et al., 2005). As climate change intensifies extreme weather events and causes broader temperature fluctuations in freshwater (FW) ecosystems, this ability may be challenged to a greater extent (Déry et al., 2024). In teleost fishes, the regulation of salt and water balance relies on the coordinated functions of the gill, skin, gastrointestinal (GI) tract, and kidney. Among these, the gill epithelium plays a crucial role in the physiological response of fishes to environmental perturbation because it is directly exposed to water. Therefore, the fish gill epithelium acts as a tissue barrier between the environment (water) and the internal fluids of the organism, and as a barrier it manages solute movement either across cells (via the transcellular pathway) or between cells (through the paracellular pathway) (Evans et al., 2005; Chasiotis et al., 2012). While there are numerous studies on transcellular solute movement across the gill epithelium, considerably less is known about solute movement through the paracellular pathway (Chasiotis et al., 2012). In vertebrates, including fishes, paracellular solute transport is primarily governed by the epithelial tight junction (TJ) complex, which includes various TJ proteins, and notably a large group of claudin (Cldn) TJ proteins (Chasiotis et al., 2012; Kolosov et al., 2013). In addition, while temperature-induced changes in salt and water balance and transcellular ion transport within the gill epithelium have been documented, the effect/s of temperature on the paracellular pathway of the gill epithelium is unexplored. Moreover, understanding sex-specific differences in these responses is equally important, as males and females may exhibit varying levels of physiological plasticity and adaptation to suboptimal temperatures.

1.2 Osmoregulation in freshwater (FW) teleost fishes

FW is a hypotonic environment relative to the body fluids (e.g. blood) of the aquatic animals that reside within it, and FW organisms exhibit strategic physiological adaptations to address the ionic/osmotic challenges that come from this (Smith, 1930; Evans et al., 2005). In FW, fishes counteract passive loss of ions and excessive water uptake across epithelial surfaces directly exposed to water by (1) active ion uptake

across the gills and ion acquisition from food by the gastrointestinal tract (to help replace solutes lost passively), (2) the production of large volumes of dilute urine by the kidney (to eliminate excess water), as well as (3) renal conservation of ions already in body fluids through reabsorption mechanisms and (4) generally maintaining tight, low permeability conditions across epithelia of the gill and integument (Evans et al., 2005; see Fig. 1). When environmental conditions change rapidly, the physiological mechanisms that balance salt and water movement must respond accordingly.

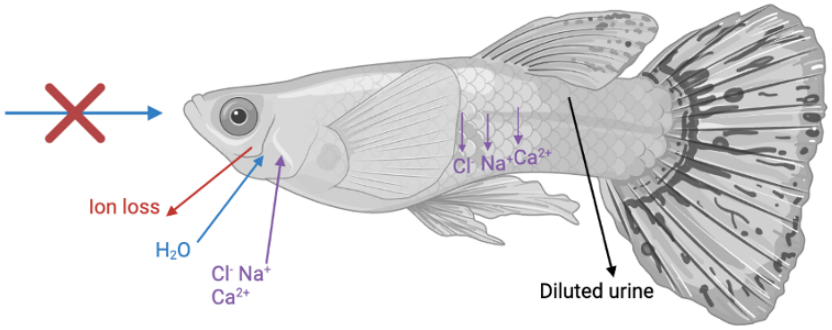

Figure 1. An overview of osmoregulatory strategies in freshwater (FW) teleosts. Fishes residing in FW are hypertonic relative to the environment. To combat ion loss, they drink little water and produce copious amounts of dilute urine. The GI tract acquires salts through dietary intake, and the kidney actively reabsorbs ions [(Evans et al., 2005; Grosell, 2010; Takvam et al., 2021); Created with BioRender.com].

1.2.1 The osmoregulatory function of the gills in FW

The epithelium of the fish gill is a highly dynamic and complex tissue composed of various cell types that include mucous and neuroepithelial cells. But the bulk of the epithelium is made up of respiratory pavement cells (PVCs), which comprise approximately 90% of the epithelial surface, and ionocytes, which are typically referred to as mitochondria-rich cells (MRCs) (Evans et al., 2005; Chasiotis et al., 2012).

Apicolateral TJs that link the cells of the FW fish gill epithelium are relatively impermeable, and this mitigates paracellular ion loss from blood to the surrounding environment (Evans et al., 2005). However, some ions are still lost to the water, and to compensate for this, transcellular transport mechanisms across MRCs facilitate ion uptake (see Fig. 2). A H^+-ATPase actively pumps H^+ ions outward across the apical membrane, generating an electrochemical gradient that drives Na^+ entry via an apical acid-sensing ion channel (ASIC)-like mechanism (Dymowska et al., 2014). Chloride (Cl^-) is absorbed through an apical Cl^-/HCO_3^- exchanger. Subsequently, Na^+ exits across the basolateral membrane via Na^+/K^+-ATPase (NKA), while K^+ is recycled through a basolateral channel. Finally, Cl^- exits the basolateral membrane through a ClC channel. (Evans et al., 2005; Tang et al., 2010).

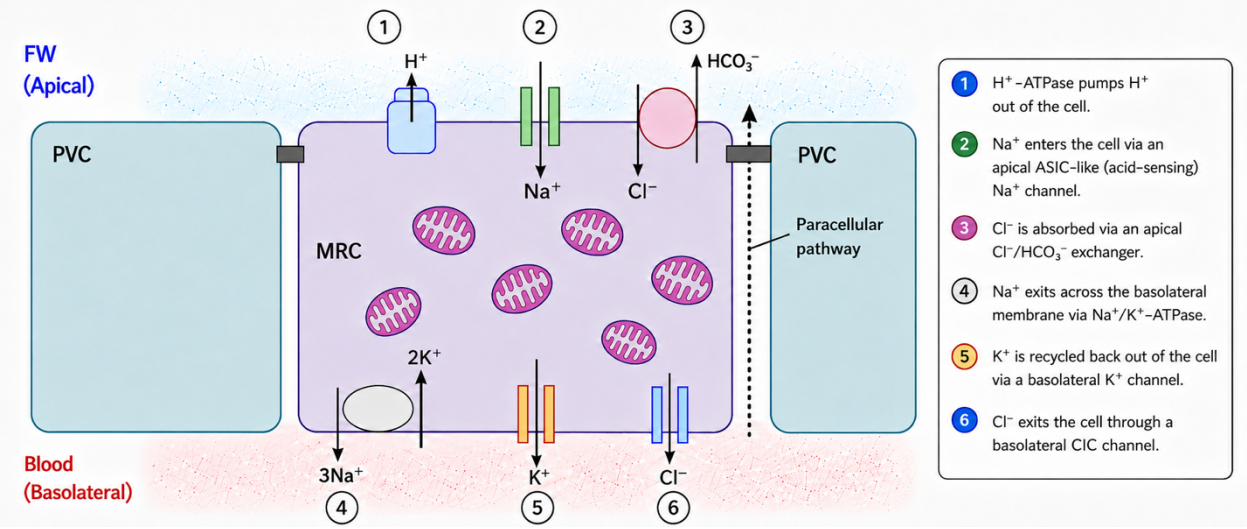

Figure 2. Proposed mechanisms of ion uptake across a mitochondria-rich cell (MRC) in the freshwater fish gill epithelium. Apical H^+ secretion generates an electrochemical gradient that can facilitate Na^+ uptake through an ASIC-like pathway, while Cl^- uptake occurs through apical Cl^-/HCO_3^- exchange. Basolateral Na^+/K^+-ATPase maintains the electrochemical gradients required for ion uptake, with K^+ recycling through K^+ channels and Cl^- subsequently transported across the basolateral membrane through a ClC channel. Tight junctions between mitochondria-rich cells and pavement cells (PVCs) restrict passive paracellular ion loss, thereby supporting net ion uptake from freshwater [(Evans et al., 2005; Tang et al., 2010; Dymowska et al., 2014); Created with Biorender.com].

1.3 The vertebrate TJ complex

In vertebrate epithelia, the TJ plays a critical role in (1) maintaining epithelial integrity and (2) regulating solute movement across epithelial barriers. In other words, TJs function both as a “fence,” which maintains apicobasal polarity by preventing the intermixing of apical and basolateral membrane components, and as a selective “gate” that regulates paracellular solute movement between adjacent cells (Gonzalez-Mariscal et al., 2003; Chasiotis et al., 2012). TJs are composed of numerous TJ proteins, and these organize into fibril-like networks at sites of cell-cell contact, appearing as interconnected strands under freeze-fracture electron microscopy (Claude and Goodenough, 1973). These belt-like structures encircle the apical region of epithelial cells and "seal" the intercellular space, thereby controlling the movement of solutes between cells through the paracellular pathway (see Fig. 3). Despite their name, TJs are not completely impermeable and instead function as selectively permeable barriers. Depending on physiological or environmental conditions, the TJ barrier may be “tight” and restrict solute movement, or “leaky” and facilitate the selective movement of solutes.

The TJ complex is composed of both transmembrane and cytosolic proteins. Cytosolic proteins connect transmembrane TJ proteins to the actin cytoskeleton and provide structural support to the junctional complex. Transmembrane proteins, including Ocln and members of the Cldn superfamily, are essential for bridging the intercellular space between epithelial cells and are major determinants of TJ permeability (Furuse et al., 1998; Chasiotis et al., 2012). Early studies demonstrated that epithelia with fewer TJ strands were generally “leakier,” whereas epithelia with more extensive strand networks exhibited tighter barriers (Claude and Goodenough, 1973). However, later work revealed that differences in epithelial permeability can occur even in tissues with similar TJ strand organization, highlighting the importance of specific TJ protein composition in determining barrier properties (Stevenson et al., 1989; Furuse et al., 2001). Therefore, TJ permeability is strongly influenced by the molecular composition and configuration of TJ proteins that collectively make up the TJ complex.

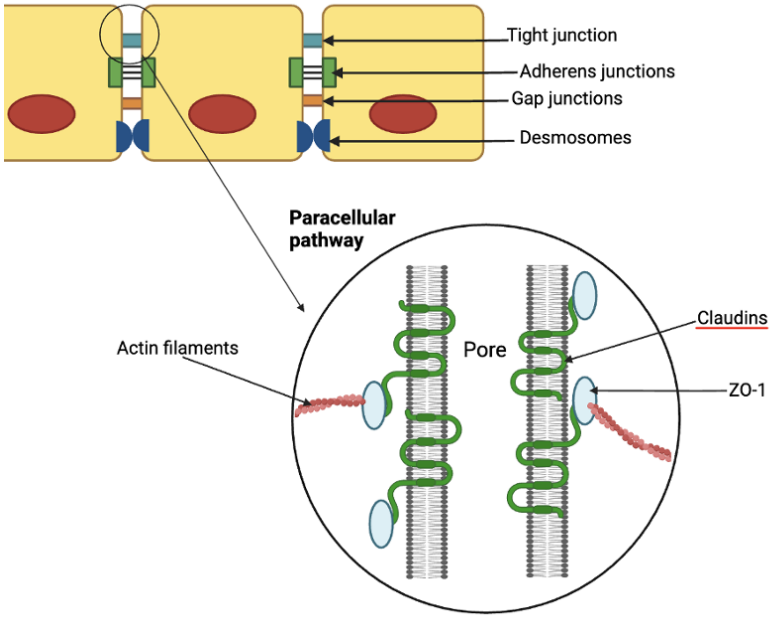

Figure 3. A generalized model of the TJ complex between epithelial cells and the paracellular pathway. Tetraspan transmembrane claudins play a direct role in regulating the permselectivity characteristics of the TJ. They accomplish this by bridging the intercellular space through their extracellular domains, forming the TJ barrier. ZO-1 contributes structural support to TJs by connecting the transmembrane TJ proteins to the actin cytoskeleton [adapted from (Vedula et al., 2009; created with Biorender.com].

1.3.1 Tight Junction Proteins

Since the identification of the first TJ proteins in the 1980s, more than 40 TJ-associated transmembrane and cytoplasmic proteins have been characterized in vertebrate epithelial and endothelial tissues (Stevenson et al., 1986; Gonzalez-Mariscal et al., 2003). In addition to their role in barrier formation, many TJ-associated proteins contribute to a variety of additional cellular processes. Certain proteins are involved in TJ assembly and maintenance, including zonula occludens-1 (ZO-1) (Fanning, 2006). Others participate in immune cell migration, cell signaling, regulation of gene expression, cell differentiation, and cell proliferation (Balda and Matter, 2000; Guillemot and Citi, 2006; Buchert et al., 2009). These findings highlight the multifunctional nature of the TJ complex and its importance in maintaining epithelial integrity and cellular homeostasis. Among TJ-associated proteins, Ocln, Cldn, and the cytoplasmic scaffolding

protein ZO-1 have received considerable attention because of their direct roles in forming and regulating the TJ barrier (Furuse et al., 1993; Furuse et al., 1998; Gonzalez-Mariscal et al., 2003).

1.3.2 Occludin

Ocln was the first transmembrane TJ protein identified and isolated from vertebrate epithelial tissues (Furuse et al., 1993). Ocln is a tetraspan transmembrane protein composed of intracellular amino- and carboxyl-terminal domains and two extracellular loops that interact between adjacent epithelial cells (Furuse et al., 1993). Following its discovery, Ocln was shown to localize specifically to TJ fibrils at sites of cell-cell contact in a variety of epithelial and endothelial tissues, supporting its role as a key structural component of the TJ complex (Furuse et al., 1993; Fujimoto, 1995; Saitou et al., 1997). Numerous studies have demonstrated the importance of Ocln in regulating TJ barrier function and paracellular permeability. Overexpression of Ocln in MDCK epithelial cells enhanced epithelial barrier integrity and was associated with greater TJ strand complexity (McCarthy et al., 1996). Conversely, reductions in Ocln abundance increased epithelial permeability to paracellular tracer molecules, indicating compromised barrier function (Kevil et al., 1998). In the mammalian nephron, Ocln abundance is greater in "tight" distal tubules compared to more permeable proximal segments, further supporting its association with enhanced epithelial barrier properties (Kwon et al., 1998; Gonzalez-Mariscal et al., 2000).

Ocln is also an important component of the TJ in teleost epithelia, including the gill, where regulation of paracellular permeability is essential for maintaining ion and water balance. Teleost fishes can possess multiple *ocln* isoforms, and their expression varies among tissues and in response to environmental conditions. For example, *ocln-a* and *ocln-b* have been identified in zebrafish, with *ocln-a* expressed in the gill and responsive to environmental conditions affecting ion balance (Kumai et al., 2011). Importantly, Ocln in fish is also responsive to endocrine regulation. Cortisol treatment of cultured rainbow trout gill epithelia increased *ocln* transcript abundance while simultaneously increasing transepithelial resistance and reducing paracellular permeability, linking Ocln regulation with corticosteroid-mediated

strengthening of the branchial epithelial barrier (Kelly and Chasiotis, 2011). In teleost fishes, cortisol is an important regulator of branchial ionoregulatory processes, acting through corticosteroid receptors expressed in the gill, including the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) (McCormick et al., 2008; Takahashi and Sakamoto, 2013). Together, these findings indicate that, as in mammalian epithelia, Ocln contributes to the regulation of epithelial barrier properties in fishes and may be dynamically regulated in response to environmental and physiological challenges.

Altered Ocln abundance has also been linked to pathological conditions characterized by impaired epithelial barrier integrity. Reduced Ocln abundance has been reported in several gastrointestinal disorders, including Crohn's disease, ulcerative colitis, and irritable bowel syndrome, suggesting that disruption of Ocln-mediated TJ function contributes to disease-associated barrier dysfunction (Gassler et al., 2001; Zeissig et al., 2007; Coëffier et al., 2010). Together, these findings support a central role for Ocln in maintaining TJ integrity and regulating selective paracellular permeability across epithelial tissues.

1.3.3 Claudin

Cldns are a large family of transmembrane tetraspan TJ proteins that play a central role in regulating paracellular permeability. Similar to Ocln, Cldns possess intracellular amino- and carboxyl-terminal domains and two extracellular loops; however, they share little sequence homology with Ocln (Furuse et al., 1998; Gonzalez-Mariscal et al., 2003; Hua et al., 2003). Cldns are capable of independently forming extensive TJ strand networks, highlighting their importance in TJ assembly and barrier formation (Furuse et al., 1998). Multiple *cldn* isoforms are expressed in epithelial and endothelial tissues in a tissue-specific manner and interact between adjacent cells to regulate the permeability characteristics of the TJ complex (Furuse et al., 1999; Daugherty et al., 2007; Krause et al., 2008). The extracellular loops of Cldns are highly variable and are major determinants of their selective permeability properties, particularly with respect to ion charge selectivity (Colegio et al., 2003; Krause et al., 2008). As a result, different Cldns can either facilitate or restrict the paracellular movement of ions and solutes. In addition to their role in barrier formation, a substantial body of research supports the role of Cldns in forming charge-selective pores within

the TJ complex (Krause et al., 2008). For example, modifications to the extracellular loop of Cldn-15 altered paracellular ion selectivity (Colegio et al., 2002). Similarly, overexpression of *cldn2* increased Na^+ permeability in MDCK cells, whereas reduced Cldn-2 abundance decreased Na^+ permeability, supporting its role as a pore-forming Cldn (Amasheh et al., 2002; Van Itallie et al., 2003; Hou et al., 2006). In contrast, overexpression of *cldn3* enhanced epithelial barrier properties and reduced paracellular permeability to ions and other solutes, consistent with a barrier-forming role for this TJ protein (Milatz et al., 2010). Likewise, Cldn-8 has been shown to reduce paracellular cation permeability when overexpressed in epithelial cell lines (Yu et al., 2003).

The *cldn* gene family is particularly diverse in teleost fishes, in part reflecting gene duplication events during teleost evolution. For example, 56 *cldn* genes have been identified in the genome of the pufferfish (*Takifugu rubripes)*, compared with approximately 27 CLDNs recognized in mammals, with 32 of the 56 teleost *cldn* genes reported to be expressed in the gill (Loh et al., 2004; Kolosov et al., 2013). Evidence indicates that teleost Cldns can exhibit both barrier- and pore-forming properties. For example, Cldn-30 from Atlantic salmon (*Salmo salar*) has been functionally characterized as a barrier-forming Cldn, with its expression reducing epithelial conductance and paracellular permeability to monovalent cations (Engelund et al., 2012). Zebrafish Cldn-15 has been associated with increased paracellular permeability, supporting a pore-forming function (Bagnat et al., 2007; Kolosov et al., 2013). In the gill, members of the Cldn-3 and Cldn-8 families have been associated with barrier-forming properties, whereas Cldn-10 isoforms have been proposed to form selective paracellular pathways for cations (Kolosov et al., 2013).

Together, these findings demonstrate that cldns can generally be classified as either barrier-forming proteins that restrict paracellular solute movement or pore-forming proteins that facilitate selective ion transport. Due to the diversity of the cldn family and their ability to form various interactions within the TJ complex, cldns are considered major determinants of tissue-specific permeability properties across epithelial and endothelial tissues (Loh et al., 2004; Krause et al., 2008).

1.3.4 Zonula occludens-1

ZO-1 was the first TJ-associated protein identified (Stevenson et al., 1986) and it remains one of the most extensively studied components of the TJ complex. ZO-1 is a cytoplasmic scaffolding protein located along the intracellular surface of the TJ, where it contains multiple binding domains that interact with transmembrane TJ proteins such as Ocln and Cldns, as well as components of the actin cytoskeleton and various intracellular signaling molecules (Fanning et al., 1998; Itoh et al., 1999; Wittchen et al., 1999; Bauer et al., 2010). Through these interactions, ZO-1 is thought to serve both structural and regulatory functions within the TJ complex. Structurally, ZO-1 links Ocln and Cldns to the actin cytoskeleton, helping to stabilize and organize the TJ barrier (Stevenson and Begg, 1994). Functionally, ZO-1 is also involved in intracellular signaling pathways associated with TJ permeability, gene expression, cell proliferation, and cellular differentiation, allowing communication between the extracellular environment and the cell interior (Van Itallie et al., 2009; Bauer et al., 2010).

Compared with Cldn and Ocln, relatively little is known about the regulation of ZO-1 in fishes. ZO-1 is expressed in teleost epithelia, including the gill, where it is expected to retain its conserved role as a cytoplasmic scaffolding protein linking transmembrane TJ proteins to the actin cytoskeleton (Chasiotis et al., 2012). Several studies have examined *zo-1* transcript abundance in response to environmental challenges, with responses varying among species and stressors (Wang et al., 2015; Cheng at al., 2021; Beghin et al., 2024). Although *zo-1* expression can be altered under some conditions, its comparatively stable expression in many experimental contexts may reflect the fundamental structural role of ZO-1 in maintaining TJ organization.

1.4 Temperature impacts on osmoregulatory function in fishes

Temperature is a key environmental factor that shapes the physiology, behaviour, and distribution of organisms by influencing fundamental biochemical and physiological processes (Christensen et al., 2021; Déry et al., 2024). Ongoing climate change is having pronounced effects on FW ecosystems, with impacts varying across climates, regions, and habitat types (Maberly et al., 2020; Paukert et al., 2021).

Water temperatures are increasing globally, with some areas experiencing particularly rapid warming, a trend further intensified by human-induced thermal pollution (Maberly et al., 2020). These changes are associated with more frequent and extreme weather events, such as intense rainfall and heatwaves, which can disrupt FW ecosystems (Polazzo et al., 2022). Projections indicate that river temperatures will continue to rise overall, although seasonal variability may occur due to shifts in snowmelt timing and precipitation patterns (Maberly et al., 2020). For FW fishes, altered temperature regimes can affect multiple physiological systems, including processes involved in maintaining ion and water balance, thereby increasing the physiological challenges associated with rapidly changing environmental conditions (Whitney et al., 2016). The present study did not examine climate change directly over the extended timescales on which it occurs. Instead, the two-week temperature exposures were used to investigate the physiological effects of sustained low- and high-temperature conditions. Such exposures may provide insight into how fishes respond to periodic thermal extremes, which are projected to increase in intensity and duration under climate change (Woolway et al., 2021).

For example, as summarized in Table 1, temperature stress alters osmoregulatory function in both FW and marine fishes through changes in transcellular ion transport processes. Under elevated temperature, common carp exhibit increased gill NKA activity, reflecting enhanced enzyme kinetics and osmoregulatory capacity (Metz et al., 2003). In contrast, heat stress can also impair osmoregulatory performance, as observed in wild-caught pink and sockeye salmon, where NKA protein activity was reduced under elevated temperature conditions (Jeffries et al., 2013). At the opposite end of the temperature spectrum, cold conditions can also impact salt and water balance in fishes. For example, in rainbow trout, acute cold exposure reduces Na^+ uptake, indicating impaired osmoregulatory function, while chronic exposure results in compensatory increases in uptake to restore osmotic balance (Gonzalez and McDonald, 2000). In contrast, common shiner shows reduced Na^+ uptake under both acute and chronic cold exposure without compensation, leading to decreased osmoregulatory capacity (Gonzalez and McDonald, 2000). Similarly, Nile tilapia exposed to cold stress exhibit decreased plasma Na^+ and Cl^- concentrations, with an initial

increase in NKA activity followed by stabilization over time, reflecting adjustments to maintain osmotic homeostasis (Li et al., 2023). In Atlantic cod, cold acclimation is associated with increased gill NKA activity, suggesting enhanced osmoregulatory capacity at low temperatures (Staurnes et al., 1994).

These findings underscore the significant impact of temperature on salt and water balance in fishes and in particular how temperature impacts elements of the transcellular epithelial transport pathways that are involved in maintaining homeostasis. But temperature-induced changes in salt and water balance, such as altered ion circulation and transcellular ion transport endpoints (e.g., NKA activity), may also impact or be influenced by alterations in the properties of the paracellular transport pathway, potentially through changes in TJ protein abundance. However, to the best of my knowledge, this potential connection between temperature changes and TJ complex properties in fishes has not yet been investigated. For FW fish specifically, it is plausible that temperature-induced alterations in passive ion loss would necessitate enhanced barrier properties in the gill TJ complex to mitigate excess ion efflux rates.

Table 1. Summary of temperature-induced physiological responses across various species of fishes. Elevated and cold stress conditions impact key osmoregulatory mechanisms, including Na+/K+-ATPase (NKA) activity, plasma osmolality, ion concentrations, and gill permeability. Findings suggest temperature-dependent variations in responses (Staurnes et al., 1994; Gonzalez and McDonald, 2000; Metz et al., 2003; Jeffries et al., 2012; Tang et al., 2014; Li et al., 2023).

Species	Temperature Condition	Key Findings
Blue-green damselfish (*Chromis viridis*)	Elevated	Increased plasma osmolality and decreased muscle water content; reduced gill NKA activity (Tang et al., 2014)
Common carp (*Cyprinus carpio*)	Elevated	Increased NKA activity (Metz et al., 2003)
Pink and sockeye salmon	Elevated	Increased plasma Cl- under heat stress (Jeffries et al., 2012)
Rainbow trout (*Oncorhynchus mykiss*)	Acute cold exposure followed by	Decreased Na+ uptake, compensatory increase in Na+ uptake under chronic cold stress (Gonzalez and McDonald, 2000)

	chronic cold exposure	
Common shiner (*Notropis cornutus*)	Acute cold exposure followed by chronic cold exposure	Decreased Na+ uptake, no compensatory increase under chronic cold stress (Gonzalez and McDonald, 2000)
Tilapia (*Oreochromis niloticus*)	Cold stress	Decreases in plasma Na+ and Cl- concentrations; initial NKA activity increase followed by stabilization (Li et al., 2023)
Atlantic cod (*Gadus morhua*)	Cold stress	Higher gill NKA activity at 1°C compared to 8°C (Staurnes et al., 1994)

1.4.1 Temperature-induced changes in TJ integrity

While the effects of temperature on TJ physiology in fishes remain unexplored, some insights can be drawn from studies conducted on mammalian models. For instance, research on rats has shown that elevating the core body temperature to 42.5°C causes rapid damage to the intestinal epithelial surface. This includes sloughing of the epithelial layer, cell death, and increased intestinal permeability (Dokladny et al., 2016). Additional studies using cell culture models revealed that hyperthermia leads to increased TJ permeability (Dokladny et al. 2006). Experiments with Madin-Darby canine kidney epithelial (MDCK) cells demonstrated that even a modest temperature increase of 1.3°C caused rapid, but reversible changes in epithelial barrier integrity (Dokladny et al. 2006). Heat stress in various cell lines, including porcine renal epithelial cells (LLC-PK1), human colon adenocarcinoma cells (Caco-2), and MDCK cells, resulted in a reduction in transepithelial resistance, which returned to baseline levels within 24 hours post-exposure (Dokladny et al. 2016). Notably, the impact of heat stress on TJ permeability was dose-dependent, with higher temperatures causing greater permeability (Dokladny et al., 2006). Regarding specific TJ proteins, Dokladny et al. (2006) observed increased protein abundance of intestinal OCLN and CLDN3 after 24 hours of heat exposure in pigs. Similarly, Price et al. (2013) found that cold exposure at 4°C elevated CLDN2 expression in the intestines of mice. These mammalian studies suggest that temperature

fluctuations influence TJ permeability and protein abundance, with increases in OCLN, CLDN2, and CLDN3 being associated with thermal stress (Dokladny et al., 2016; Price et al., 2013).

1.5 Heat shock proteins and thermal stress in fishes

Heat shock proteins (HSPs) are a highly conserved family of molecular chaperones that play a central role in maintaining cellular homeostasis under conditions of environmental stress. Among these, HSP70 and HSP90 are particularly important in responding to thermal challenges, as they assist in protein folding, prevent aggregation of denatured proteins, and facilitate the repair or degradation of damaged proteins (Feder and Hoffman, 1999). Increased HSP abundance in response to temperature change is widely recognized as a hallmark of cellular stress, particularly in response to elevated temperature but also in response to challenges to salt and water balance (Deane et al., 2002). In fish, temperature is a critical environmental variable that directly influences physiological processes, including metabolism, ionoregulation, and epithelial function (Kültz, 2005; Iwama et al., 1998). The gill is especially sensitive to thermal stress due to its role as the primary interface between the organism and the external environment, as well as its function in gas exchange and ion transport (Evans et al., 2005). Exposure to elevated temperatures can disrupt membrane integrity, alter enzyme activity, and impair ion transport mechanisms within gill epithelial cells. Consequently, the upregulation of HSPs in gill tissue is thought to serve a protective function by stabilizing cellular proteins and preserving epithelial structure and function (Feder and Hofmann, 1999; Iwama et al., 1998).

In addition to their role in protein homeostasis, HSPs have been implicated in the maintenance of epithelial barrier integrity (Iwama et al., 1998). For example, HSP70 has been shown to mitigate stress-induced damage to TJs and reduce oxidative stress and inflammation, thereby helping to preserve barrier function under adverse conditions (Dokladny et al., 2016). This is particularly relevant in the gill epithelium, where TJs regulate paracellular permeability and are essential for maintaining ion balance.

Therefore, HSP induction may represent a key compensatory mechanism that supports both cellular integrity and ionoregulatory function in fish exposed to thermal stress (Dokladny et al., 2016).

1.6 Sex-specific differences in mammalian tight junction proteins

Findings from Orr et al. (2019) demonstrate that environmental factors, such as exposure to silver nanoparticles, can significantly and differentially influence the abundance of CLDN and other TJ genes in the intestines of rats. Specifically, females showed significant upregulation of TJ proteins like CLDN1, -5, -6, and -10 in response to AgNP exposure, while males exhibited minimal or downregulated responses (Orr et al., 2019). These differences suggest that TJ integrity and its regulation by environmental factors may be sex-specific. Thus, these sex-specific differences seen in Cldn/*cldn* abundance under environmental stress in mammalian epithelial tissue provide a rationale for studying similar mechanisms in fish, given their shared presence of Cldn proteins. If fish exhibit comparable responses to environmental factors like temperature changes, it could reveal sex-specific variations in their ability to maintain ion balance and barrier integrity.

1.7 Poeciliids and sex-specific differences in temperature tolerance

Poeciliid fishes are useful model organisms for laboratory-based investigations of sex-specific differences in salt and water balance under conditions of temperature change. This is because species such as *Poecilia reticulata* are euryhaline, eurythermal, easy to maintain/breed under controlled conditions and they exhibit clear sexual dimorphism. Traits such as the presence of the male gonopodium, a modified anal fin for internal fertilization, and differences in body shape/colour, makes sex differentiation straightforward. Indeed, *Poecilia reticulata* exhibit sex-specific differences in thermal tolerance, which may have implications for their ecological performance, reproductive output, and responses to climatic variability (Muñoz et al., 2012). These distinctions often reflect underlying differences in reproductive roles, energy allocation, and physiological plasticity (Reznick and Endler, 1982; Muñoz et al., 2012). In *Poecilia reticulata*, females consistently demonstrate higher critical thermal maxima (CT_{Max}) compared to males

(Chung, 2001). In the studies of Chung (2001) CT_{Max} values ranging from 38.95°C to 40.33°C were reported for males and 39.48°C to 40.61°C for females. The critical thermal minimum (CT_{Min}) for this species has been recorded at 9.41°C, below which metabolic and osmoregulatory function may become severely impaired (Yanar et al., 2019).

1.8 Overarching objectives and hypothesis

Considering that (1) temperature can affect salt and water balance in fish, partly through alterations in the transcellular transport pathway of the gill epithelium, (2) changes in the transcellular pathway are likely to influence the paracellular pathway of the gill epithelium, (3) temperature can modulate the vertebrate TJ complex, and (4) male and female fish may display sex-specific differences in osmoregulatory responses to environmental changes such as temperature, *I hypothesize that barrier TJ proteins in the FW fish gill will exhibit sex-specific alterations in abundance in sub-optimal temperatures as the gill epithelium reorganizes to mitigate paracellular ion loss*.

2. MATERIALS AND METHODS

2.1 Experimental animals

All experimental procedures were conducted in accordance with the guidelines of the Canadian Council for Animal Care and were approved by the York University Animal Care Committee. Male and female guppies (*Poecilia reticulata*) were obtained from a local supplier and initially housed in 60 L glass aquaria within the York University aquatic vivarium facilities. Water conditions were as follows: Water was dechlorinated municipal tap water. System water was continuously aerated and biofiltered and a photoperiod of 12h light:12h dark was maintained throughout the husbandry period. Water temperature was 25°C. Water quality parameters (nitrite, nitrate, and ammonia) were monitored every other day using an API® FW master test kit to ensure stability within acceptable limits. Individuals were fed twice daily with commercial feed (Hikari® Tropical Fancy Guppy semi-floating granules), receiving a total daily ration equivalent to approximately 1.8–2% of the total fish biomass in each tank. Prior to experimental manipulation, and to control for the reproductive status of the female fish, salinity was gradually increased from FW to 15‰ brackish water to avoid the stress associated with an abrupt salinity transfer. All fish were exposed to brackish water (15‰ Instant Ocean Sea Salts in FW) for one week to induce regression of pregnancy in females. This was undertaken to minimize any potential confounding effects of reproductive status on osmoregulatory and thermal responses.

Following a week in brackish water, fish were gradually returned to FW under previously described conditions. Once FW conditions were restored, fish were randomly assigned to one of six experimental groups based on sex and temperature treatment: (1) male, 33°C; (2) female, 33°C; (3) male, 25°C; (4) female, 25°C; (5) male, 17°C; and (6) female, 17°C. The medium temperature (25°C) represented the standard maintenance condition, whereas 17°C and 33°C represented suboptimal low- and high-temperature conditions, respectively, selected to induce temperature stress without causing acute mortality. To reach the "High" (33°C) and "Low" (17°C) experimental temperature regimes, water temperature in each tank was adjusted gradually over a one-week period. Fish held at 25°C were considered controls and

were designated “Medium”. Fish were then maintained at their respective temperatures for an additional two-week acclimation period prior to collection of samples. Temperature conditions were maintained using appropriate equipment for each treatment. Low-temperature aquaria were chilled using Aqua Logic Delta Star Chillers, whereas medium (25°C) and high (33°C) treatments were regulated using EHEIM thermocontrol e 150 submersible aquarium heaters.

2.2 Fish dissection

P. reticulata were euthanized via immersion in an overdose solution of buffered tricaine methanesulfonate (MS-222). Following euthanasia, whole-body wet mass was recorded using a Mettler Toledo AE163 analytical balance. Then the head was removed, and the operculum was carefully opened to expose the gill arches. Gills were excised and immediately transferred into 1.5 mL microcentrifuge tubes. Gills destined for molecular analyses were placed in 0.5 mL of ice-cold TRIzol reagent. All samples were rapidly frozen on dry ice and subsequently stored at −80°C until further processing.

2.3 Carcass moisture, ion content and gill enzyme activity

To determine carcass moisture content, the carcass (i.e. the body excluding the head and all organs contained within the visceral cavity), was weighed and then dried at 60°C for one week, after which dry mass was recorded. Briefly, carcass samples were placed into pre-weighed tubes and weighed to obtain wet mass, then dried at 60°C to a constant mass. Dry mass was subsequently recorded and used to calculate tissue moisture content (Chasiotis and Kelly, 2008). Carcass moisture content (%) was calculated using the equation:

$$\text{Carcass moisture content (\%)} = \frac{Wet\ body\ weight - Dry\ body\ weight}{Wet\ body\ weight} \text{ x } 100$$

For ion analysis, dried carcass samples were processed following established protocols (Al-Jandal and Wilson, 2011). Carcass chloride concentrations were measured using a colorimetric assay adapted from

Zall et al. (1956). Samples and standards were run in duplicate on a 96-well microplate and absorbance was read at 480 nm.

Gill Na^+/K^+-ATPase (NKA) activity was measured following established protocols (McCormick, 2011), with a modified ouabain concentration. Ouabain (5.0 mM) was used as a specific inhibitor to distinguish NKA activity from total ATPase activity. Protein concentrations of the supernatant were determined using a Bradford assay with bovine serum albumin (BSA) as a standard (Chasiotis and Kelly, 2008).

2.4 RNA extraction

Gill tissues frozen in TRIzol reagent were defrosted on ice, transferred to clean plastic tubes and homogenized on ice using a PRO250 electric homogenizer (ProScientific) for approximately 10 seconds. To facilitate phase separation, samples were incubated at room temperature for 5 minutes to allow dissociation of nucleoprotein complexes. Chloroform was then added at a ratio of 0.2 mL per 1 mL of TRIzol, and tubes were vigorously shaken for 15 seconds before a 2-minute incubation at room temperature. Samples were subsequently centrifuged at 13,000 rpm for 15 minutes at 4°C, resulting in separation into aqueous, interphase, and organic layers, with RNA localized in the upper aqueous phase.

The aqueous phase was carefully transferred to a new nuclease-free microcentrifuge tube, avoiding disturbance of the interphase. RNA was precipitated by the addition of isopropanol (0.5 mL per 1 mL of TRIzol), followed by gentle inversion and incubation at room temperature for 10 minutes. Samples were then centrifuged at 13,000 rpm for 10 minutes at 4°C to pellet the RNA. The resulting pellet was washed with 1 mL of chilled 75% ethanol and centrifuged at 7,500 × g for 5 minutes at 4°C. After removal of ethanol, the pellet was briefly air-dried and resuspended in 5–50 µL of nuclease-free water, depending on pellet size, with a 10-minute incubation at room temperature to ensure complete dissolution. RNA concentration and purity were quantified using a Thermo Fisher Scientific Multiskan SkyHigh microplate spectrophotometer.

2.5 cDNA synthesis

Prior to cDNA synthesis, residual genomic DNA was removed using the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad). iScript DNase Buffer and Reverse Transcription Supermix were thawed at room temperature and subsequently maintained on ice. RNA samples (≤1 μg) were combined with nuclease-free water to a final volume of 14 μL in nuclease-free PCR tubes. To each sample, 1.5 μL of DNase Buffer and 0.5 μL of DNase were added, yielding a total reaction volume of 16 μL. Where appropriate, a DNase master mix was prepared and aliquoted to ensure consistency across samples.

Samples were incubated at 25°C for 5 minutes to facilitate genomic DNA digestion, followed by 75°C for 5 minutes to inactivate DNase. Tubes were then returned to ice. For reverse transcription, 4 μL of Reverse Transcription Supermix was added to each sample, bringing the final reaction volume to 20 μL. cDNA synthesis was carried out using the following thermal profile: 25°C for 5 minutes (primer annealing), 46°C for 20 minutes (reverse transcription), and 95°C for 1 minute (enzyme inactivation). Synthesized cDNA samples were stored at −30°C until further analysis.

2.6 Quantitative real-time PCR

Transcript abundance of target genes was quantified using quantitative real-time PCR (qRT-PCR) in both experimental and baseline expression samples. Reactions were performed using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada) in combination with gene-specific primers (Table 2). Amplification was conducted on a CFX Connect™ Real-Time PCR Detection System with Maestro software (Bio-Rad). Thermal cycling conditions consisted of an initial polymerase activation and denaturation step at 95°C for 30 seconds, followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 55–60°C for 20 seconds (see Table 2 for target genes and primer information). To verify amplification specificity, melt curve analysis was performed following each run (65–95°C, 0.5°C increments). Primer efficiency was assessed by generating standard curves using five-point serial dilutions of cDNA. Only reactions with acceptable amplification

efficiencies and threshold cycle (Ct) values within the dynamic range were included in subsequent analyses. Relative transcript abundance was calculated using the Pfaffl method (Pfaffl, 2001), which accounts for gene-specific amplification efficiencies. All target gene expression was normalized to 18S rRNA, which did not vary significantly among experimental groups and was therefore validated as a stable reference gene.

Table 2. Primer sequences, annealing temperatures and accession numbers used for quantitative real-time PCR (qRT-PCR). Genes encoding claudin (Cldn) tight junction proteins, occludin (*ocln*) isoforms, zonula occludens-1 (*zo-1*), mineralocorticoid receptor (*mr*), heat shock proteins (*hsp70* and *hsp90*), and the reference gene 18S in *Poecilia reticulata*. Primer sequences are shown in the 5′–3′ direction. Primer sequences were designed and confirmed by Helen Chasiotis and Eleni Diakanatasis.

Gene	Primer	Primer Sequence	Annealing Temperature (°C)	Accession Number
cldn3b	Forward	ATAATCCCCGTGTCCTGGTC	60	Pending
	Reverse	CGTAGTCTCTCCTGTCATAGCC		
cldn3c	Forward	GATGGGTTTGGAGATTGTGG	55	PQ198788
	Reverse	TGCGAAGTGATGATGTTGG		
cldn3d	Forward	CAGATTGTGCCCGTTTCCTG	55	PQ198789
	Reverse	TTAGTGCCTTCTCCTCCTGTGG		
cldn8a	Forward	GAACTGTGGGAAGGTCTGTG	55	Pending
	Reverse	GGCTGGGAGAATCAATAATG		
cldn8b	Forward	GTGTTGCTCTCTGGCTCTGG	60	PQ198790
	Reverse	CACAGGAATAAGGTCACAAATGC		
cldn8c	Forward	ACCCGTGTCTTGGACTGGTC	60	PQ198791
	Reverse	GGATGCTTGGAATGCTGGAG		
cldn8d	Forward	CAAGCCACCAATGTCCTATGC	60	Pending
	Reverse	TCCCAGCGAGTTTCCATCAC		
cldn10a	Forward	TGTGGAAAATGTGCGTGACTG	60	PQ198792
	Reverse	AGACGAGCCTTGGTGGTGTC		
cldn10b	Forward	TGCTCACTTTCTGCCTGTTCC	60	PQ198793
	Reverse	GCTTCGTCTGCCCTCTTGTG		
cldn10c	Forward	GGGTGGATTTTGGTCTGTTCC	60	Pending
	Reverse	TGATGGAGGTGATGATGAGAGC		
cldn10d	Forward	GGTGCTGCTGTTTTCATTGG	60	PQ198786

	Reverse	CATACGAAGGTGTCCCGATTC		
cldn10e	Forward	TAGGTGGAATGGGTGGTTCG	60	Pending
	Reverse	GCAGGGAAATCATAGCAGTTGG		
cldn10f	Forward	CATTTCTGGGTTTGGTGGGATG	60	PQ198794
	Reverse	CATTGAGTCGGGAGGGTTGG		
cldn15a	Forward	ATCACCACCTCCACCATCTACG	55	Pending
	Reverse	CTCCACCCGCTTTTGAACAC		
cldn15b	Forward	TGATTGCTGTGTCTTGGTATGC	60	PQ198795
	Reverse	AGCGTTCCTGCCGTAGTTG		
cldn28a	Forward	CTGGGGTTCTGGTGTTGGTC	60	PQ198796
	Reverse	TAGATTTGGCGACGGATGTG		
cldn28b	Forward	GATGCTCAGAAGAGAGAGTTGG	55	PQ198797
	Reverse	TATTGCTGCTTGTTGGTGTC		
cldn28c	Forward	CAGGTGTTTTGGGAAGGTC	55	PQ198798
	Reverse	GTCGTAGCGGCAGAAGTTG		
cldn30a	Forward	CATCGCTCTTCATCGGTTG	60	Pending
	Reverse	GTCTTTGGGAGGGCAGTTG		
cldn30b	Forward	GGAGAAGGCTAAGGCGAAGG	60	Pending
	Reverse	AAACGGGAACGAGGCAGAG		
cldn30c	Forward	AAAGGTCAAGGTGGGCATTG	60	PQ198799
	Reverse	AAAGTTCTGGATGATGGTGTTGG		
cldn30d	Forward	TCAGTTCGCTATGTGGTAGTCC	55	Pending
	Reverse	ATGGGTTTGATGGGTTGAG		
cldn31	Forward	CCTTTACCTCGTTCCTGTCTGC	60	Pending
	Reverse	GGGACTGCTTTCTCCACTGC		
ocln-a	Forward	ACCCCTCATCCCAGACATC	55	PQ198801
	Reverse	ACCAAGAAGCCGAACACAAC		
ocln-b	Forward	GCACAATACCAGACACAGACTCAGG	60	PQ198802
	Reverse	TTCACCCGACACATTCTTCACC		
zo-1	Forward	AAACCCAGACACCAAACAGG	60	Pending
	Reverse	CTCCAAACCAACCGTCATTC		
mr	Forward	AACCTGGGAGCACGGAAGTC	60	Pending
	Reverse	CTGTGGCAATCTGGGCATC		
hsp70	Forward	AACGGTGTGTTTGAAGTGGTAGC	60	Pending
	Reverse	TTGGCACGGGTTAGGGTCTC		
hsp90	Forward	CTTTGACCCTGACCGACACG	60	Pending

	Reverse	CCCACACCGAACTGACCAATC		
18s	Forward	CGATTCTGTGGGTGGTGGTG	60	Pending
	Reverse	AGTGGGGTTCAGCGGGTTAC		

2.7 Statistical analysis

All data are presented as mean ± SEM (n), where *n* represents the number of biological replicates per treatment group. Data were assessed for normality using the Shapiro–Wilk test, and potential outliers were identified using Grubbs' test in conjunction with Q–Q plot inspection. Where necessary, data were transformed to meet assumptions of normality. To evaluate the effects of temperature and sex, as well as their interaction, data were analyzed using a two-way analysis of variance (ANOVA), followed by Tukey post hoc tests for multiple comparisons. When data were pooled across sex and the effect of temperature was considered independently, data were analyzed using a one-way ANOVA followed by Tukey's post hoc test for multiple comparisons when a significant overall effect was detected. A significance threshold of $P \leq 0.05$ was applied throughout. All statistical analyses were performed using GraphPad Prism (version 9.2.0; GraphPad Software Inc., La Jolla, CA, USA).

3. RESULTS

3.1 Expression profile of TJ protein genes in the gill

Gill transcript expression profiling in male guppies maintained at the control temperature (25 °C) revealed the presence of several predicted barrier-forming TJ *cldns*, including members of the *cldn-8, -28,* and *-30* families. Among the *cldns* examined, *cldn-8c* and *-8d* exhibited the greatest relative transcript abundance, while *cldn-28a* and *-28b* were also relatively abundant. The TJ-associated genes *ocln* and *zo-1* were also detected, with *ocln-a* exhibiting greater transcript abundance than *ocln-b* and *zo-1*. Mineralocorticoid receptor (*mr*) transcript was also present in the gill but at a lower abundance than several of the more highly expressed *cldn* isoforms (see Fig. 4).

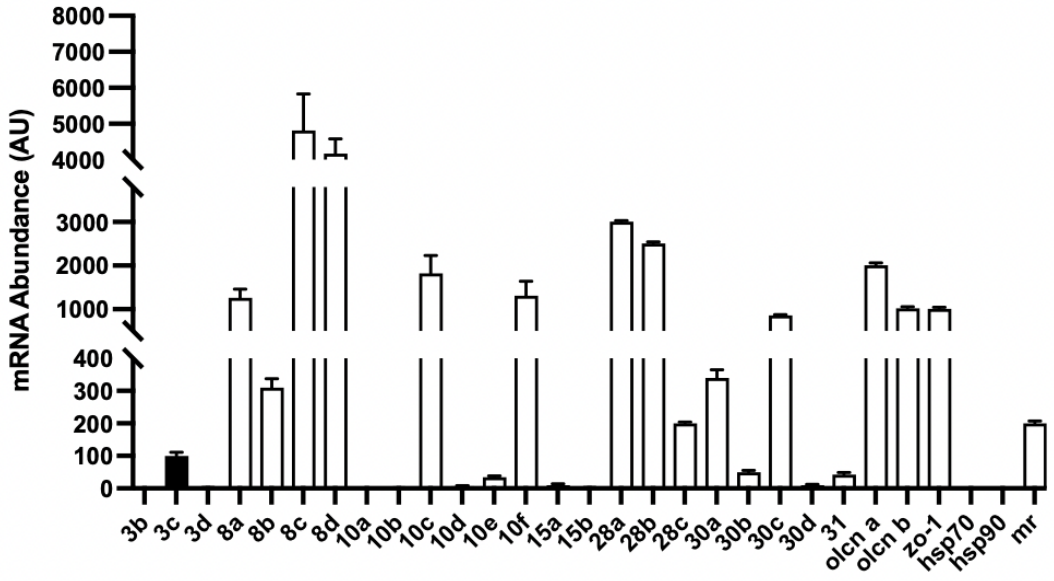

Figure 4. Expression profile of mRNA encoding tight junction (TJ) proteins in the gill of the male guppy (*Poecilia reticulata*). Transcript abundance was normalized using the reference gene 18S and data are presented relative to *cldn3b* which is assigned a value of 100 (represented by the black bar). All data are expressed as mean values ± SEM (n = 8), and are generated using male fish held in a control temperature of 25 °C.

Table 3. Summary of two-way ANOVA results examining the effects of temperature, sex, and their interaction on whole-animal physiological variables and gill transcript abundance in guppies (*P. reticulata*). "Yes" indicates a statistically significant effect ($P \leq 0.05$), whereas "No" indicates a non-significant effect ($P > 0.05$).

Response variable	Temperature effect	Sex effect	Temperature and sex interaction
Body moisture	No (p = 0.0735)	No (p = 0.2177)	No (p = 0.3404)
Carcass Cl^- content	Yes (p = 0.0133)	No (p = 0.3420)	No (p = 0.7404)
NKA activity	No (p = 0.1030)	Yes (p = 0.0148)	No (p = 0.3430)
cldn3b	Yes (p <0.0001)	No (p = 0.8830)	No (p = 0.8288)
cldn3c	No (p = 0.5365)	No (p = 0.8144)	No (p = 0.5990)
cldn3d	No (p = 0.2987)	No (p = 0.5804)	No (p = 0.7663)
cldn8a	No (p = <0.0001)	No (p = 0.9395)	No (p = 0.9990)
cldn8b	Yes (p <0.0001)	Yes (p = 0.0004)	Yes (p <0.0001)
cldn8c	Yes (p <0.0001)	Yes (p = 0.0045)	Yes (p = 0.0002)
cldn8d	Yes (p <0.0001)	Yes (p = 0.0444)	Yes (p = 0.0059)
cldn10a	No (p = 0.4448)	No (p = 0.7429)	No (p = 0.0640)
cldn10b	No (p = 0.7661)	Yes (p = <0.0001)	No (p = 0.3899)
cldn10c	No (p = 0.4732)	No (p = 0.9825)	No (p = 0.7681)
cldn10d	No (p = 0.9028)	Yes (p = 0.0270)	No (p = 0.4910)
cldn10e	No (p = 0.6111)	No (p = 0.4760)	No (p = 0.1765)
cldn10f	No (p = 0.5486)	No (p = 0.7944)	No (p = 0.8331)
cldn15a	Yes (p = 0.0281)	Yes (p = 0.0226)	No (p = 0.3664)
cldn15b	Yes (p = 0.0325)	No (p = 0.1272)	No (p = 0.8863)
cldn28a	No (p = 0.9751)	No (p = 0.8223)	No (p = 0.7715)
cldn28b	No (p = 0.3446)	No (p = 0.3829)	No (p = 0.4238)
cldn28c	No (p = 0.7354)	No (p = 0.6233)	No (p = 0.5071)
cldn30a	Yes (p = 0.0030)	No (p = 0.0761)	No (p = 0.1252)
cldn30b	Yes (p = 0.0028)	No (p = 0.8796)	No (p = 0.9226)
cldn30c	No (p = 0.7775)	No (p = 0.8731)	No (p = 0.8717)
cldn30d	Yes (p <0.0001)	No (p = 0.3581)	No (p = 0.9629)
cldn31	Yes (p <0.0001)	No (p = 0.2991)	No (p = 0.3884)
ocln-a	No (p = 0.3446)	No (p = 0.3829)	No (p = 0.4238)
ocln-b	No (p = 0.4968)	No (p = 0.3953)	No (p = 0.0864)
zo-1	Yes (p <0.0001)	No (p = 0.7328)	No (p = 0.9989)
mr	Yes (p <0.0001)	No (p = 0.9568)	No (p = 0.9654)

hsp70	Yes (p <0.0001)	No (p = 0.4079)	No (p = 0.9509)
hsp90	Yes (p <0.0001)	No (p = 0.5027)	No (p = 0.9769)

3.2 Heat shock proteins

Transcript abundance of *hsp* exhibited temperature-dependent variation in both male and female fish. Abundance of *hsp70* and *hsp90* was significantly elevated at high temperature relative to medium and low temperature conditions in both sexes ($p \leq 0.05$; Fig. 5A–B). In contrast, no significant differences were observed between medium and low temperature treatments. No significant differences were observed between sexes within a temperature treatment. When data were collapsed (i.e. sexes were pooled so that only the effect of temperature was considered), both *hsp70* and *hsp90* retained significant temperature-dependent differences, with highest levels of transcript abundance observed under high temperature conditions (Fig. 5C–D).

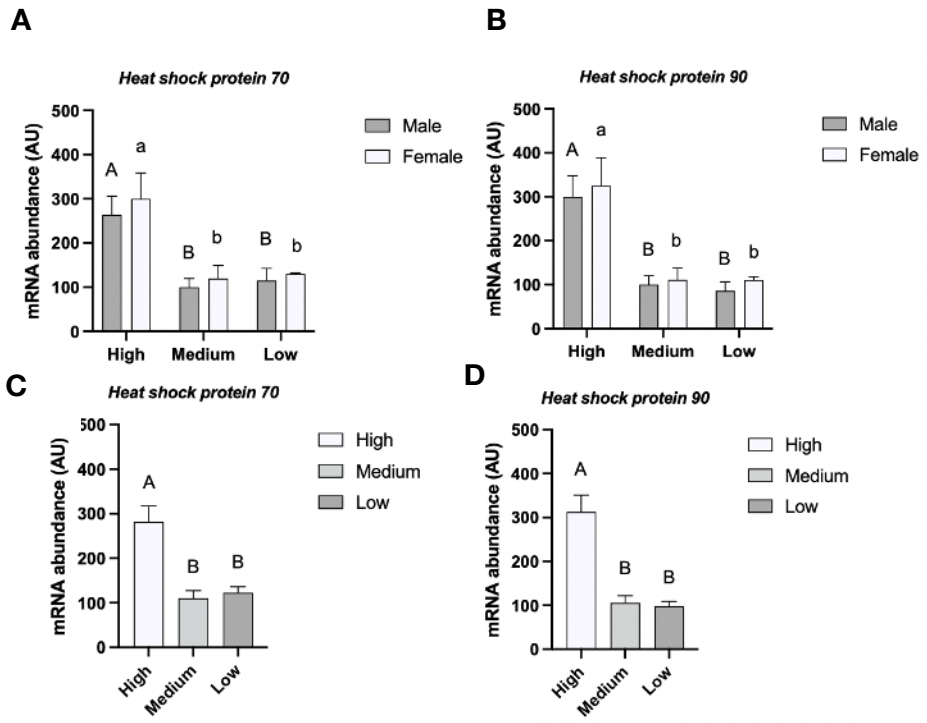

Figure 5. Transcript abundance of gill heat shock proteins 70 (hsp70) and 90 (hsp90) in male and female guppies (*Poecilia reticulata*) held in high, medium and low temperature conditions. Top panels (A–B) show sex-specific data where uppercase letters indicate significant differences between temperature groups in males, while lowercase letters indicate significant differences between temperature groups in females ($P \leq 0.05$). Bottom panels (C–D) show data collapsed across sex so that only temperature and not sex is considered. Transcript abundance was normalized using 18S and expressed relative to the medium male group (which was assigned a value of 100). Data are presented as mean ± SEM ($n = 10$ for A and B; $n = 20$ for C and D).

3.3 Carcass moisture content

Carcass moisture content did not differ significantly across temperature treatments or between sexes. When males and females were analyzed separately, no significant effects of temperature were observed in either sex. Similarly, when data were collapsed across sex, no significant differences were detected among high, medium, and low temperature groups. Two-way ANOVA revealed no significant interaction between sex and temperature and no significant main effects ($p > 0.05$; Fig. 6).

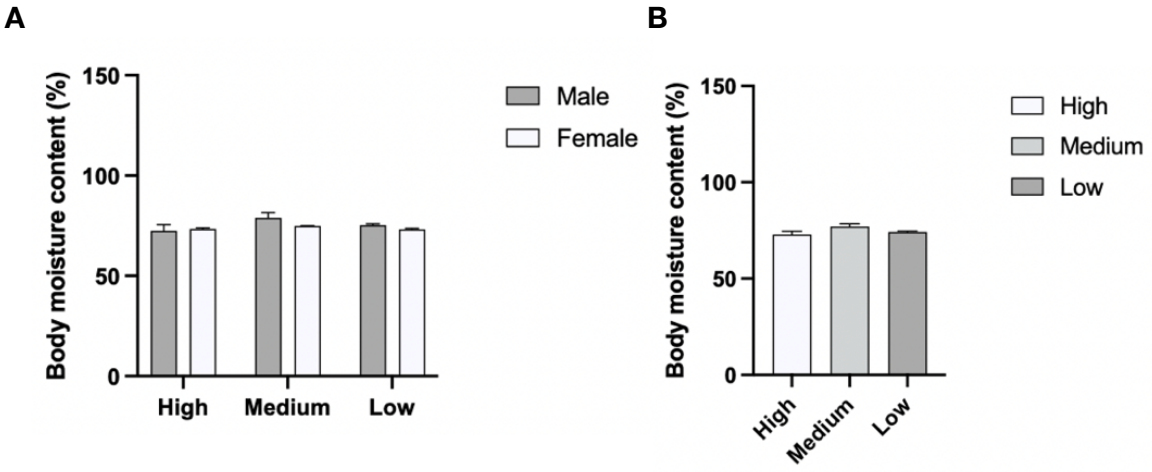

Figure 6. Carcass moisture content (%) in male and female guppies (*Poecilia reticulata*) held in high, medium and low temperature conditions. Panel (A) shows sex-specific data while panel (B) shows data collapsed across sex. Data are presented as mean ± SEM ($n = 10$ for A; $n = 20$ for B). No significant differences in body moisture content were observed between sexes or among temperature treatments (two-way ANOVA, $p > 0.05$).

3.3.1 Carcass chloride content

Carcass chloride content did not differ significantly across temperature treatments when males and females were analyzed separately, indicating no detectable temperature effect within each sex (Fig. 7A). However, when data were collapsed across sex, chloride content differed significantly among temperature treatments (one-way ANOVA, $p \leq 0.05$; Fig. 7B), revealing an overall temperature-dependent effect on ion content. This discrepancy suggests that variability within sexes may have masked treatment effects in sex-specific analyses, while pooling data increased statistical power to detect an overall temperature response. Importantly, the absence of consistent sex-specific differences indicates that males and females responded similarly to temperature in terms of whole-body ion balance.

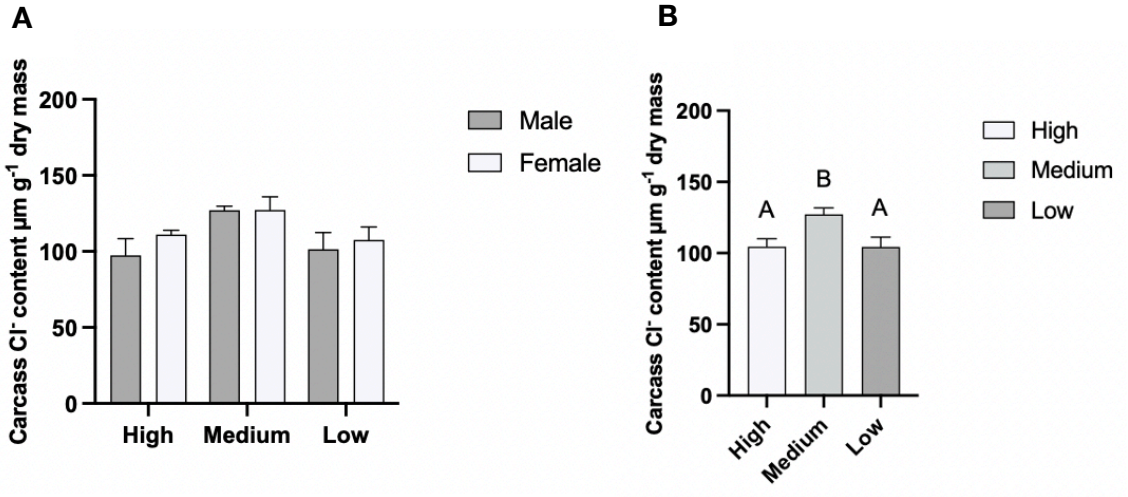

Figure 7. Carcass chloride content in male and female guppies (*Poecilia reticulata*) held under high, medium, and low temperature conditions. Panel (A) shows sex-specific data, while panel (B) shows data collapsed across sex, such that only temperature and not sex is considered. Different uppercase letters indicate significant differences between temperature groups ($P \leq 0.05$). Data are presented as mean ± SEM (n = 8–10 for A; n = 16–20 for B).

3.3.2 Gill Na^+/K^+-ATPase activity

Gill NKA activity exhibited sex-specific variation in response to low temperature. In males, NKA activity was significantly lower at low temperature relative to the medium (control) temperature, whereas no significant differences among temperature treatments were observed in females (Fig. 8A). In addition, under low-temperature conditions, NKA activity was significantly lower in males relative to females ($p \leq 0.05$; Fig. 8A), while no sex differences were observed at the medium (control) or high temperatures. When data were pooled across sex, such that only the effect of temperature was accounted for, these differences were no longer detected, with no significant differences in NKA activity observed among temperature treatments ($p > 0.05$; Fig. 8B).

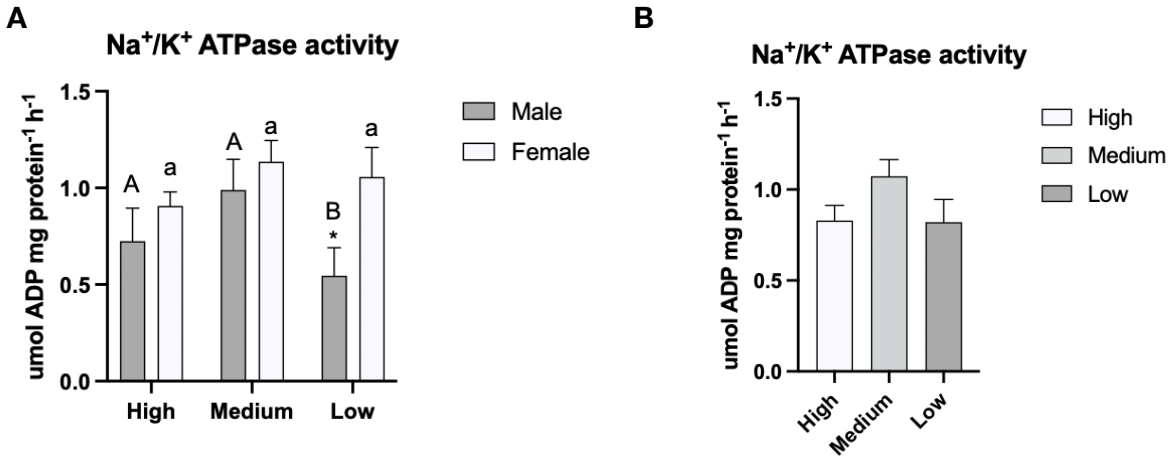

Figure 8. Na^+/K^+-ATPase (NKA) activity in the gill of male and female guppies (*Poecilia reticulata*) held under high, medium, and low temperature conditions. Panel (A) shows sex-specific data, where uppercase letters indicate significant differences between temperature groups in males, while lowercase letters indicate significant differences between temperature groups in females. An asterisk (*) indicates a significant difference between sexes within the same temperature group ($P \leq 0.05$). Panel (B) shows data collapsed across sex, such that only temperature and not sex is considered. Different uppercase letters indicate significant differences between temperature groups ($P \leq 0.05$). Data are presented as mean ± SEM ($n = 10$ for A; $n = 20$ for B).

3.4 Gill TJ protein transcript abundance

3.4.1 Isoforms of *cldn3*

Transcript abundance of select *cldn3* isoforms exhibited temperature-dependent variation in the gills of the guppy. For *cldn3b*, mRNA abundance was significantly higher at high temperature relative to medium (control) and low temperature conditions in both sexes ($p \leq 0.05$; Fig. 9A) and no difference was found between sexes. In contrast, *cldn3c* and *cldn3d* showed no significant differences across temperature treatments in either sex. When data were collapsed across sex, *cldn3b* retained significant temperature-dependent differences, while cldn3c and cldn3d remained unchanged (Fig. 9D–F).

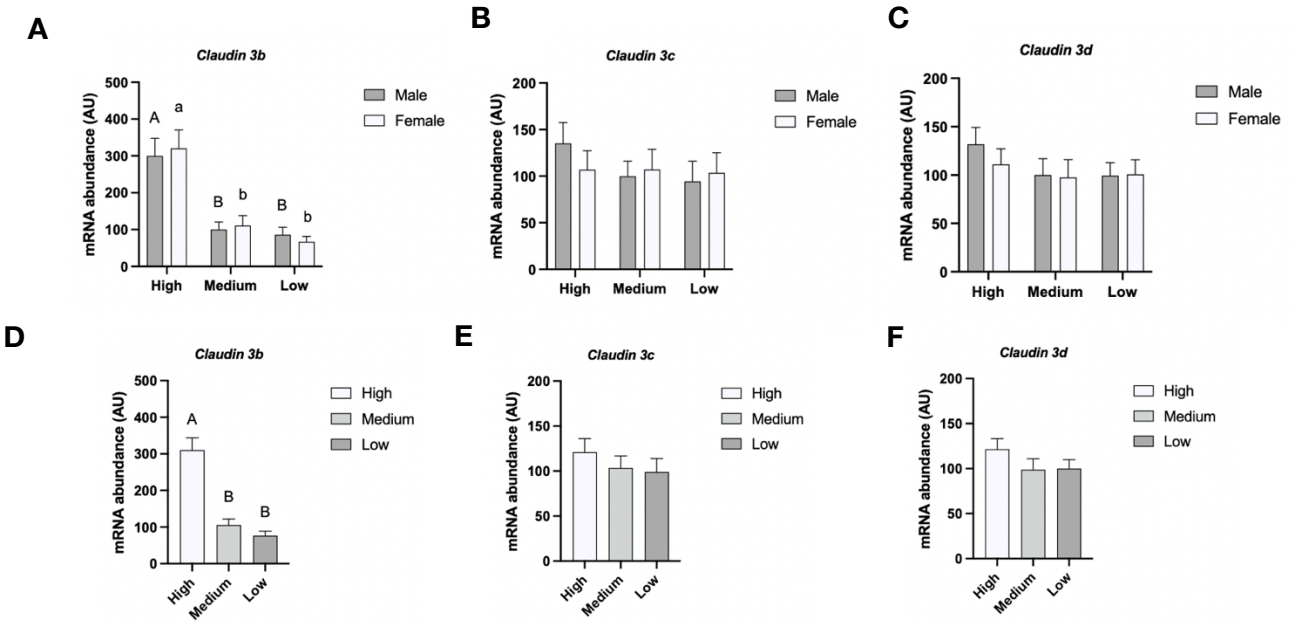

Figure 9. Transcript abundance of claudin (*cldn*) tight junction proteins (*cldn3b, -3c*, and *-3d*) in the gill of male and female guppies (*Poecilia reticulata*) held under high, medium, and low temperature conditions. Top panels (A–C) show sex-specific data, where uppercase letters indicate significant differences between temperature groups in males, while lowercase letters indicate significant differences between temperature groups in females ($P \leq 0.05$). Bottom panels (D–F) show data collapsed across sex, such that only temperature and not sex is considered. Transcript abundance was normalized to 18S and expressed relative to the medium male group. Data are presented as mean ± SEM ($n = 10$ for A–C; $n = 20$ for D –F).

3.4.2 Isoforms of *cldn8*

Several cldn8 isoforms exhibited significant temperature-dependent changes in transcript abundance. Specifically, *cldn8a* and *cldn8d* transcript abundance was significantly elevated at high temperature relative to medium and low temperature conditions in both sexes ($p \leq 0.05$; Fig. 10A, D). Interestingly, *cldn8b* and *cldn8c* also exhibited increased transcript abundance in response to elevated temperature, but in female fish only (Fig. 10B, C). In addition, the elevated *cldn8d* transcript abundance observed at high temperature in both sexes was more pronounced in female fish, leading to a significant difference between male and female fish (greater in female fish than male) for this *cldn* isoform also (Fig. 10D). A final sex-specific observation for the cldn8s was that cldn8b exhibited a significantly lower transcript abundance in low temperature water (versus control and high temperature conditions), but only in female fish (Fig. 10B).

When sexes were pooled and only the effect of temperature taken into consideration, the effect of temperature on transcript abundance of cldn8a and cldn8d was quite consistent with sex-specific observations in that both were elevated at high temperatures relative to control and low temperatures. But the female -specific responses of *cldn8b* and -*8c* were masked as was the female-specific response to elevated temperature of *cldn8d* (Fig. 10E–H).

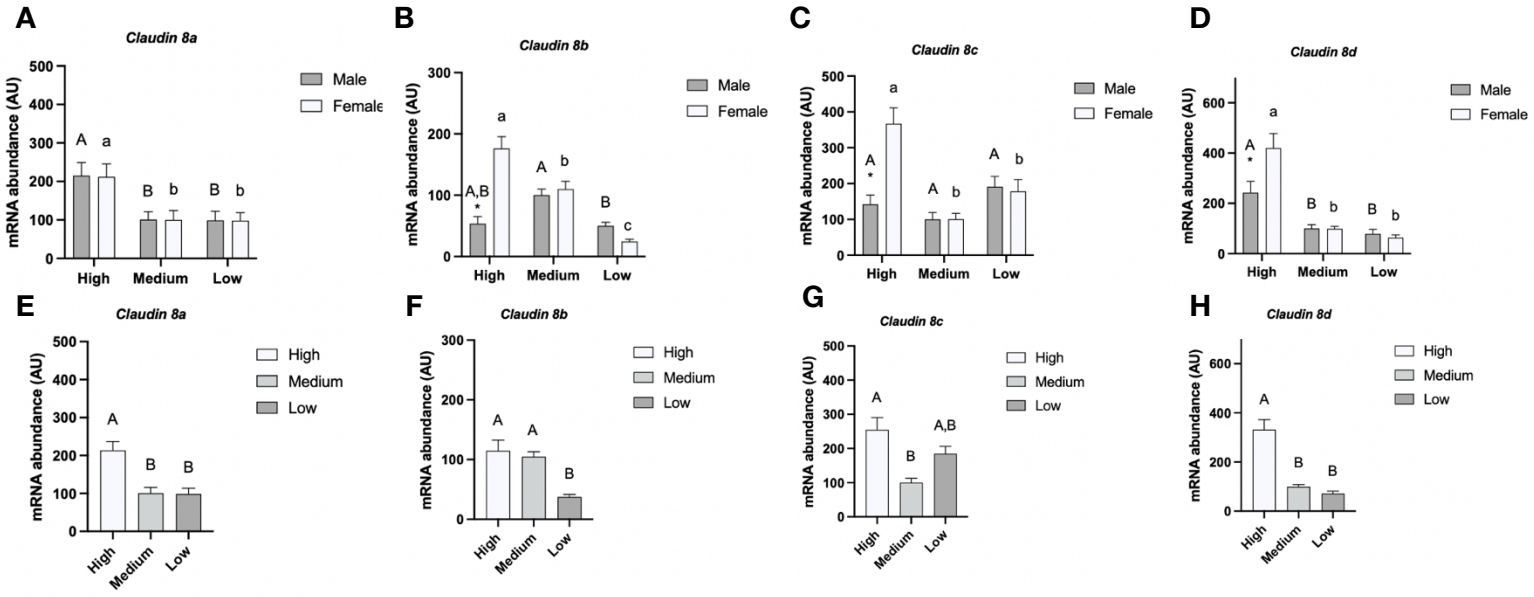

Figure 10. Transcript abundance of claudin (*cldn*) tight junction proteins (*cldn8a, -8b, -8c*, and *-8d*) in the gill of male and female guppies (*Poecilia reticulata*) held under high, medium, and low temperature conditions. Top panels (A–D) show sex-specific data, where uppercase letters indicate significant differences between temperature groups in males, while lowercase letters indicate significant differences between temperature groups in females. An asterisk (*) indicates a significant difference between sexes within the same temperature group ($P \leq 0.05$). Bottom panels (E–H) show data collapsed across sex, such that only temperature and not sex is considered. Different uppercase letters indicate significant differences between temperature groups ($P \leq 0.05$). Transcript abundance was normalized to 18S and expressed relative to the medium male group. Data are presented as mean ± SEM (n = 10 for A–D; n = 20 for E –H).

3.4.3 Isoforms of *cldn10* and *cldn28*

Transcript abundance of the *cldn10* and *cldn28* isoforms was largely unaffected by temperature. When male and female fish were examined separately, no significant differences in transcript abundance were observed among low, medium (control), and high temperature conditions for the majority of *cldn10* isoforms (Fig. 11). However, significant sex-specific differences were observed for *cldn10b* and *cldn10d*, with females exhibiting significantly higher transcript abundance than males under all three temperature conditions (Fig. 11B, D). Similarly, transcript abundance of *cldn28a*, *cldn28b*, and *cldn28c* did not differ significantly among temperature treatments in either male or female fish (Fig. 12B–D).

When data from male and female fish were pooled and only the effect of temperature was considered, no significant temperature-dependent differences were detected for any of the *cldn10* or *cldn28* isoforms examined (Figs. 11–12). Importantly, pooling the data across sex masked the sex-specific differences observed for *cldn10b* and *cldn10d*, as the consistently higher transcript abundance observed in females was no longer distinguishable when males and females were combined. While pooling did not reveal any additional effects of temperature, the sex-specific analyses identified differences in *cldn10b* and *cldn10d* transcript abundance that would otherwise have been overlooked.

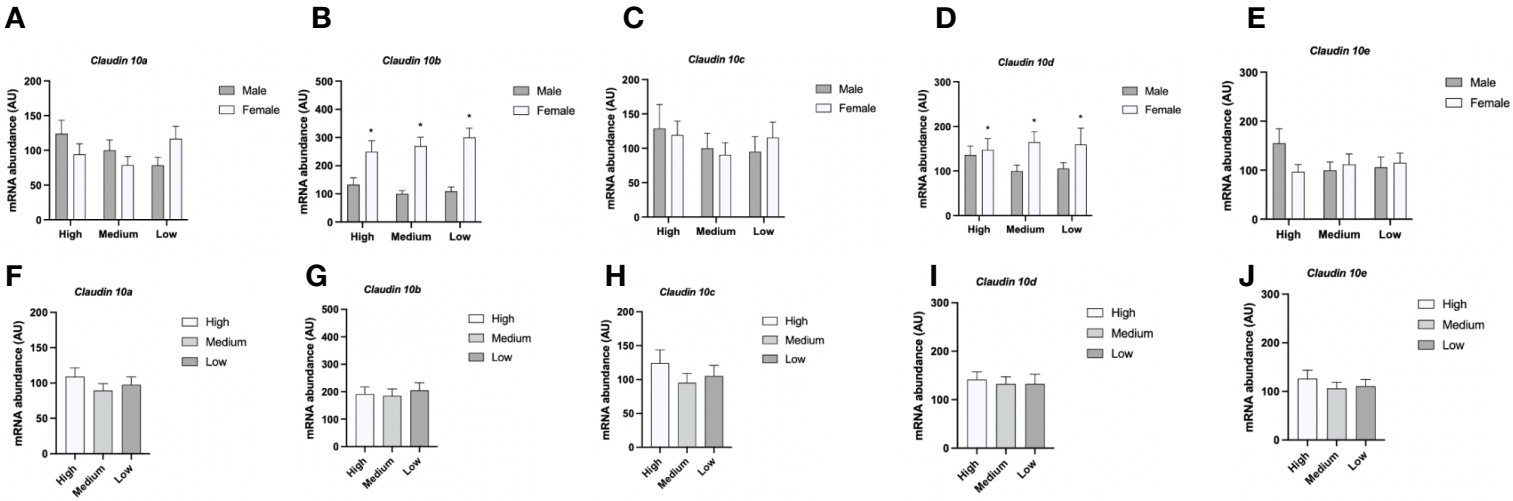

Figure 11. Transcript abundance of claudin (*cldn*) tight junction proteins (*cldn-10a, -10b, -10c, -10d,* and *-10e*) in the gill of male and female guppies (*Poecilia reticulata*) held under high, medium, and low temperature conditions. Top panels (A–E) show sex-specific data. An asterisk (*) indicates a significant difference between sexes within the same temperature group ($P \leq 0.05$). Bottom panels (F–J) show data collapsed across sex, such that only temperature and not sex is considered. Transcript abundance was normalized to 18S and expressed relative to the medium male group. Data are presented as mean ± SEM ($n = 10$ for A–E; $n = 20$ for F–J).

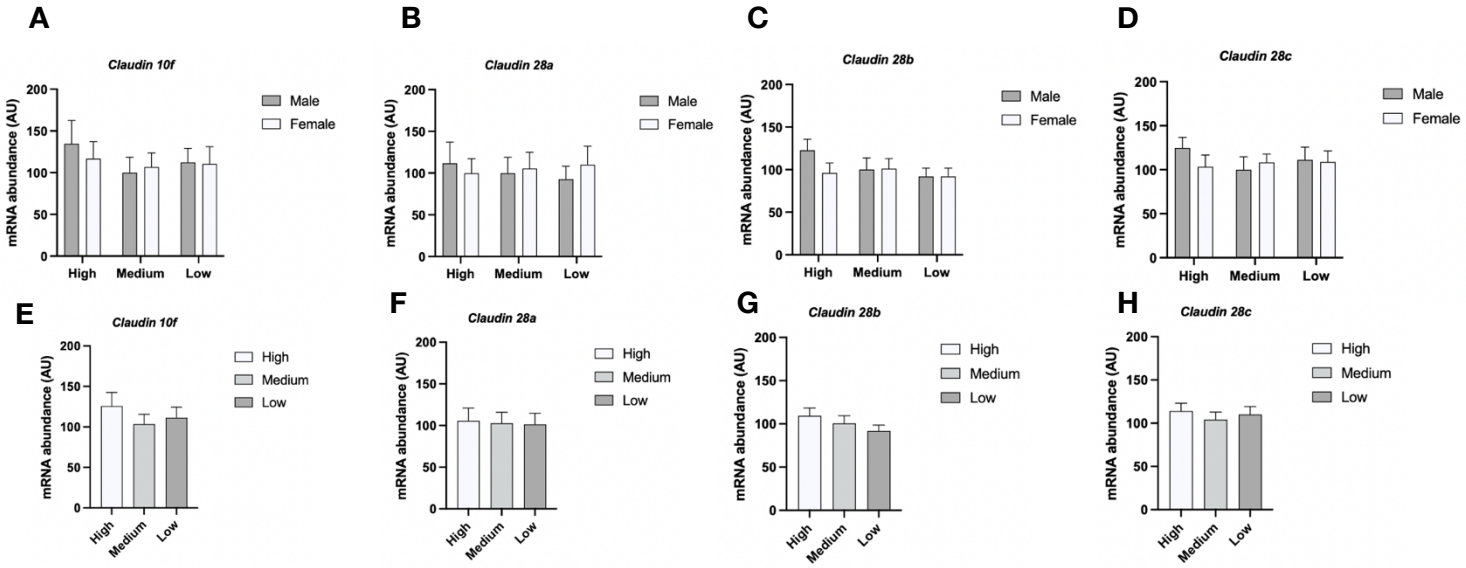

Figure 12. Transcript abundance of claudin (*cldn*) tight junction proteins (*cldn10f, -28a, -28b,* and *-28c*) in the gill of male and female guppies (*Poecilia reticulata*) held under high, medium, and low temperature conditions. Top panels (A–D) show sex-specific data. Bottom panels (E–H) show data collapsed across sex, such that only temperature and not sex is considered. No significant differences were observed between temperature groups or between sexes ($P > 0.05$). Transcript abundance was normalized to 18S and expressed relative to the medium male group. Data are presented as mean ± SEM ($n = 10$ for A–D; $n = 20$ for E –H).

3.4.4 Isoforms of *cldn15*

Transcript abundance of *cldn15a* and *cldn15b* did not differ significantly among temperature treatments when male and female fish were examined separately (Fig. 13A, B). Thus, neither high nor low temperature resulted in a significant change in transcript abundance relative to the medium (control) temperature within either sex.

When sexes were pooled and only the effect of temperature was considered, significant temperature-dependent differences became apparent for both *cldn15* isoforms. Specifically, *cldn15a* transcript abundance was significantly higher at high temperature relative to the medium temperature, whereas abundance at low temperature did not differ significantly from the medium temperature ($p \leq 0.05$; Fig. 13C). In contrast, *cldn15b* transcript abundance was significantly higher at low temperature relative to the medium temperature, whereas abundance at high temperature did not differ significantly from the medium temperature ($p \leq 0.05$; Fig. 13D).

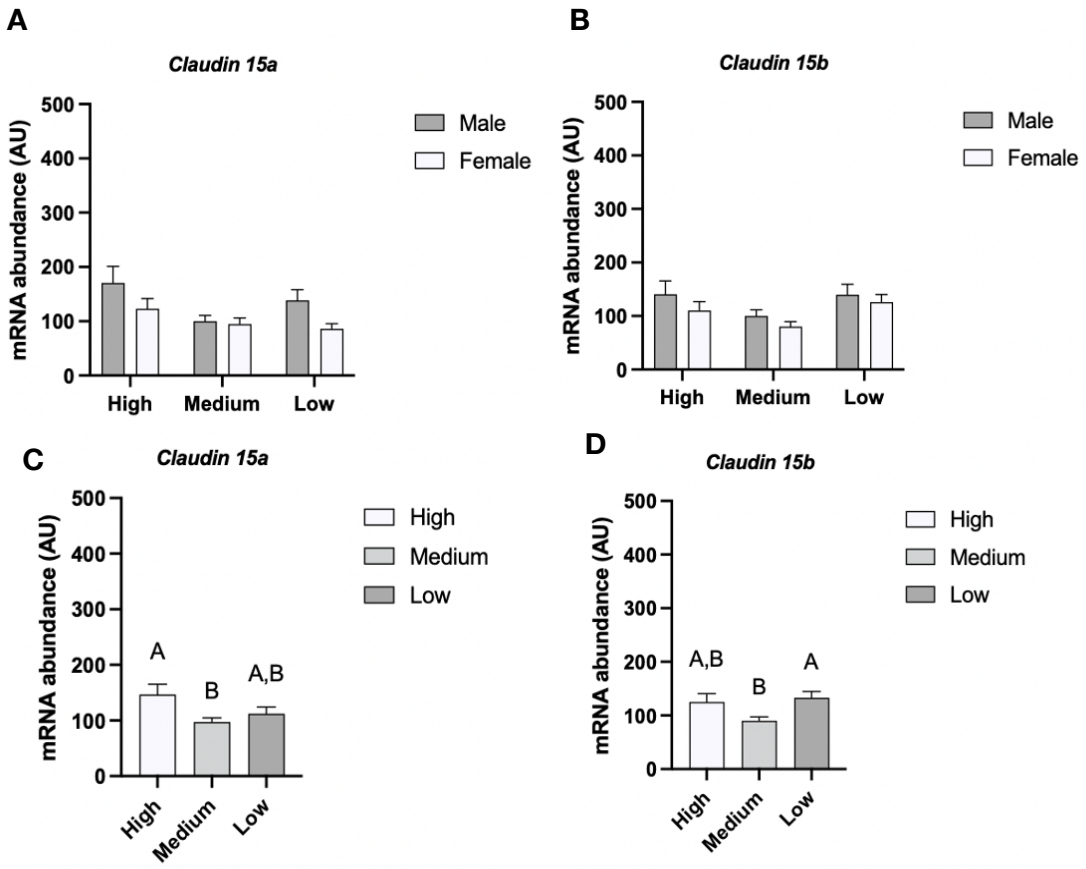

Figure 13. Transcript abundance of claudin (*cldn*) tight junction proteins (*cldn15a* and *cldn15b*) in the gill of male and female guppies (*Poecilia reticulata*) held under high, medium, and low temperature conditions. Top panels (A–B) show sex-specific data, where uppercase letters indicate significant differences between temperature groups in males, while lowercase letters indicate significant differences between temperature groups in females ($P \leq 0.05$). Bottom panels (C–D) show data collapsed across sex, such that only temperature and not sex is considered. Different uppercase letters indicate significant differences between temperature groups ($P \leq 0.05$). Transcript abundance was normalized to 18S and expressed relative to the medium male group. Data are presented as mean ± SEM ($n = 10$ for A and B; $n = 20$ for C and D).

3.4.5 Isoforms of *cldn30* and *cldn31*

Several *cldn30* isoforms exhibited significant temperature-dependent changes in transcript abundance. Specifically, *cldn30a* transcript abundance was significantly higher at high temperature relative to the medium (control) temperature in both males and females ($p \leq 0.05$; Fig. 14A), whereas transcript abundance at low temperature did not differ significantly from the medium temperature in either sex. A similar response was observed for *cldn30d*, with transcript abundance significantly higher at high temperature relative to the medium temperature in both sexes, while no significant difference was observed between low and medium temperature conditions ($p \leq 0.05$; Fig. 14D). In contrast, *cldn30b* and *cldn30c* did not exhibit significant temperature-dependent changes in either male or female fish (Fig. 14B, C). *cldn31* also exhibited a significant response to temperature in both sexes, with transcript abundance significantly higher at both high and low temperatures relative to the medium temperature ($p \leq 0.05$; Fig. 14E).

When sexes were pooled and only the effect of temperature was considered, *cldn30a* transcript abundance was significantly higher at high temperature relative to the medium temperature, whereas no significant difference was observed between low and medium temperature conditions ($p \leq 0.05$; Fig. 14F). Interestingly, pooling the sexes revealed a significant temperature-dependent response for *cldn30b* that was not detected in the sex-specific analyses, with transcript abundance significantly higher at high temperature relative to the medium temperature, while low temperature did not differ significantly from the medium temperature ($p \leq 0.05$; Fig. 14G). *cldn30c* remained unaffected by temperature when sexes were pooled (Fig. 14H). For *cldn30d* and *cldn31*, transcript abundance was significantly higher at both high and low temperatures relative to the medium temperature ($p \leq 0.05$; Fig. 14I, J).

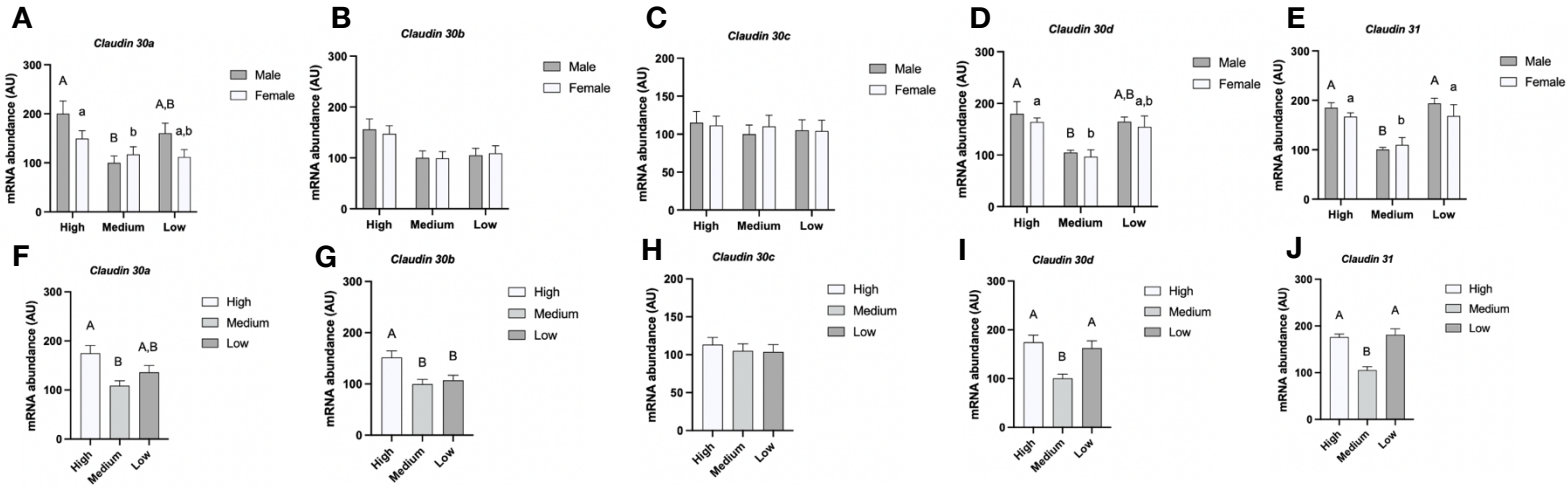

Figure 14. Transcript abundance of claudin (*cldn*) tight junction proteins (*cldn30a, -30b, -30c, -30d,* and *-31*) in the gill of male and female guppies (*Poecilia reticulata*) held under high, medium, and low temperature conditions. Top panels (A–E) show sex-specific data, where uppercase letters indicate significant differences between temperature groups in males, while lowercase letters indicate significant differences between temperature groups in females. Bottom panels (F–J) show data collapsed across sex, such that only temperature and not sex is considered. Different uppercase letters indicate significant differences between temperature groups ($P \leq 0.05$). Transcript abundance was normalized to 18S and expressed relative to the medium male group. Data are presented as mean ± SEM ($n = 10$ for A–E; $n = 20$ for F –J).

3.4.6 Isoforms of *ocln*

Transcript abundance of the two *ocln* isoforms was largely unaffected by temperature. Specifically, neither *ocln-a* nor *ocln-b* transcript abundance differed significantly between high and medium (control) temperature conditions or between low and medium temperature conditions in either male or female fish (Fig. 15A, B).

When sexes were pooled and only the effect of temperature was taken into consideration, the pattern remained consistent with the sex-specific observations. Neither *ocln-a* nor *ocln-b* transcript abundance differed significantly at high or low temperature relative to the medium (control) temperature (Fig. 15C, D). Thus, no significant temperature-dependent changes in transcript abundance were detected for either *ocln* isoform in sex-specific or pooled analyses.

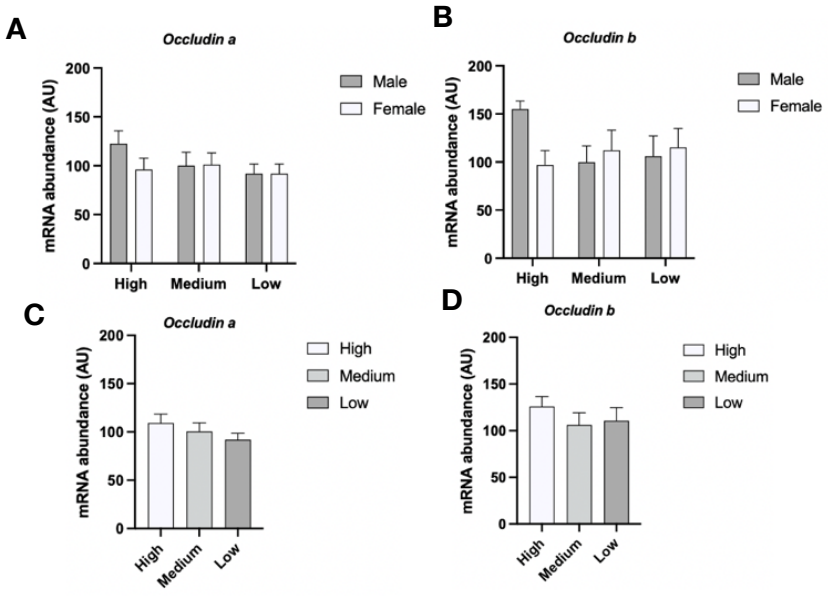

Figure 15. Transcript abundance of occludin (*ocln*) tight junction proteins (*ocln-a* and *ocln-b*) in the gill of male and female guppies (*Poecilia reticulata*) held under high, medium, and low temperature conditions. Top panels (A–B) show sex-specific data. Bottom panels (C–D) show data collapsed across sex, such that only temperature and not sex is considered. No significant differences were observed between temperature groups or between sexes ($P > 0.05$). Transcript abundance was normalized to 18S and expressed relative to the medium male group. Data are presented as mean ± SEM ($n = 10$ for A and B; $n = 20$ for C and D).

3.4.7 *zo-1*

zo-1 exhibited a significant temperature-dependent change in transcript abundance. Specifically, *zo-1* transcript abundance was significantly elevated at high temperature relative to the medium (control) temperature in both male and female fish ($p \leq 0.05$; Fig. 16A). In contrast, *zo-1* transcript abundance at low temperature did not differ significantly from the medium temperature in either sex. No significant differences in *zo-1* transcript abundance were observed between male and female fish at any of the temperature conditions examined (Fig. 16A).

When sexes were pooled and only the effect of temperature taken into consideration, the effect of temperature on *zo-1* transcript abundance was consistent with the sex-specific observations. *zo-1* transcript abundance was significantly elevated at high temperature relative to the medium (control) temperature, whereas transcript abundance at low temperature did not differ significantly from the medium temperature ($p \leq 0.05$; Fig. 16B).

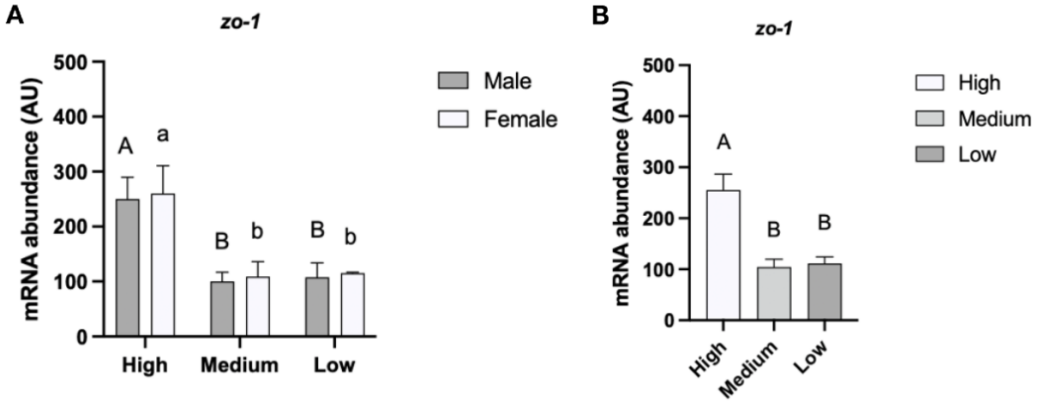

Figure 16. Transcript abundance of zonula occludens-1 (*zo-1*) in the gill of male and female guppies (*Poecilia reticulata*) held under high, medium, and low temperature conditions. Panel (A) shows sex-specific data, where uppercase letters indicate significant differences between temperature groups in males, while lowercase letters indicate significant differences between temperature groups in females ($P \leq 0.05$). Panel (B) shows data collapsed across sex, such that only temperature and not sex is considered. Different uppercase letters indicate significant differences between temperature groups ($P \leq 0.05$). Transcript abundance was normalized to 18S and expressed relative to the medium male group. Data are presented as mean ± SEM ($n = 10$ for A; $n = 20$ for B).

3.5 *mr*

mr exhibited a significant temperature-dependent change in transcript abundance. Specifically, *mr* transcript abundance was significantly elevated at high temperature relative to the medium (control) temperature in both male and female fish ($p \leq 0.05$; Fig. 17A). In contrast, *mr* transcript abundance at low temperature did not differ significantly from the medium temperature in either sex. No significant differences in *mr* transcript abundance were observed between male and female fish at any of the temperature conditions examined (Fig. 17A).

When sexes were pooled and only the effect of temperature was taken into consideration, the response of *mr* was consistent with the sex-specific observations. *mr* transcript abundance was significantly elevated at high temperature relative to the medium (control) temperature, whereas transcript abundance at low temperature did not differ significantly from the medium temperature ($p \leq 0.05$; Fig. 17B).

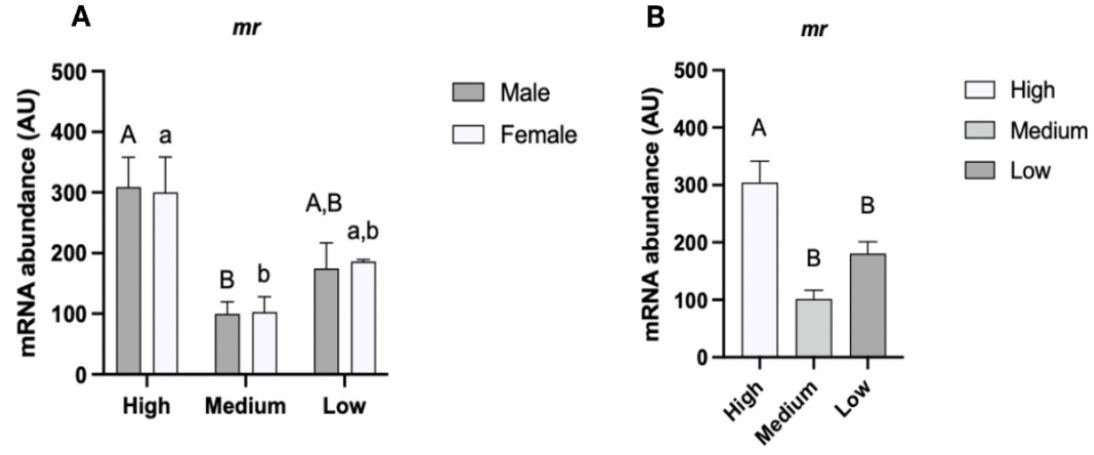

Figure 17. Transcript abundance of mineralocorticoid receptor (*mr*) in the gill of male and female guppies (*Poecilia reticulata*) held under high, medium, and low temperature conditions. Panel (A) shows sex-specific data, where uppercase letters indicate significant differences between temperature groups in males, while lowercase letters indicate significant differences between temperature groups in females ($P \leq 0.05$). Panel (B) shows data collapsed across sex, such that only temperature and not sex is considered. Different uppercase letters indicate significant differences between temperature groups ($P \leq 0.05$). Transcript abundance was normalized to 18S and expressed relative to the medium male group. Data are presented as mean ± SEM ($n = 10$ for A; $n = 20$ for B).

4. DISCUSSION

4.1 Overview

The present study investigated the effects of temperature on osmoregulatory function in *P. reticulata*, with a particular focus on the gill epithelium TJ complex and potential sex-specific responses. It was hypothesized that sub-optimal temperatures would compromise the integrity of the gill epithelium, and this would result in an increase in the abundance of barrier-forming TJ proteins so as to mitigate increased paracellular ion loss. Furthermore, it was proposed that in this response, sex-specific differences would manifest. Both contentions were supported by the data as exposure to temperature stress resulted in increased transcript abundance of several TJ-associated genes, particularly barrier-forming Cldns, and sex-specific differences were observed in some instances (see Table 4 for a summary of gene-specific responses). While not all TJ-associated genes were influenced by temperature and/or sex, the selective regulation of specific *cldns* suggested that targeted and sex-specific remodeling of the TJ complex in response to environmental temperature change can occur while systemic endpoints of salt and water balance remained relatively stable between males and females. Furthermore, in some instances sex-dependent *cldn* expression patterns within temperature treatments were masked when data were pooled. Taken together, data provide evidence that male and female *P. reticulata* may not employ identical molecular strategies to maintain epithelial barrier function under sub-optimal temperature conditions, and in particular, high temperature environments.

Table 4. Summary of temperature- and sex-specific responses of gill epithelium tight junction (TJ)-associated genes, mineralocorticoid receptor (*mr*), and heat shock protein genes in *Poecilia reticulata*. Genes are classified according to their functional role and observed response to temperature and/or sex. Temperature-dependent responses indicate significant differences among high, medium (control), and low temperature treatments, whereas sex-dependent responses indicate significant differences between males and females within or across temperature treatments. Responses detected only after sexes were pooled are indicated accordingly.

Genes	Putative classification	Response type	Observed pattern
cldn3b	Barrier	Temperature-dependent	High > medium and low in both sexes; same pattern when sexes pooled
cldn3c, cldn3d	Barrier	No response	No significant temperature or sex differences
cldn8a	Barrier	Temperature-dependent	High > medium and low in both sexes; same pattern when sexes pooled
cldn8b	Barrier	Temperature- and sex-dependent	Females: high and medium > low; no temperature effect in males or when sexes pooled
cldn8c	Barrier	Temperature- and sex-dependent	Females: increased at high temperature; no temperature effect in males or when sexes pooled
cldn8d	Barrier	Temperature- and sex-dependent	High > medium and low in both sexes; females > males at high temperature; pooled: high > medium and low
cldn10a, cldn10c, cldn10e, cldn10f	Pore	No response	No significant temperature or sex differences
cldn10b, cldn10d	Pore	Sex-dependent	Females > males at high, medium, and low temperatures; no temperature effect
cldn15a	Pore	Temperature-dependent when sexes pooled	No temperature effect within either sex; pooled: high > medium

cldn15b	Pore	Temperature-dependent when sexes pooled	No temperature effect within either sex; pooled: low > medium
cldn28a, cldn28b, cldn28c	Barrier	No response	No significant temperature or sex differences
cldn30a	Barrier	Temperature-dependent	High > medium in both sexes; low not different from medium; pooled: high > medium
cldn30b	Barrier	Temperature-dependent when sexes pooled	No temperature effect within either sex; pooled: high > medium
cldn30c	Barrier	No response	No significant temperature effect
cldn30d	Barrier	Temperature-dependent	High > medium in both sexes; pooled: high and low > medium
cldn31	Barrier	Temperature-dependent	High and low > medium in both sexes; same pattern when sexes pooled
ocln-a, ocln-b	Barrier-associated	No response	No significant temperature or sex differences
zo-1	TJ scaffolding	Temperature-dependent	High > medium in both sexes; low not different from medium; same pattern when sexes pooled
mr	Corticosteroid receptor	Temperature-dependent	High > medium in both sexes; low not different from medium; same pattern when sexes pooled

4.2 Heat shock proteins

HSPs are among the most highly conserved cellular stress proteins and are rapidly induced in response to environmental stressors, particularly elevated temperature (Feder and Hofmann, 1999; Iwama et al., 1998; Kültz, 2005). Acting as molecular chaperones, HSP70 and HSP90 stabilize newly synthesized proteins, prevent protein denaturation and aggregation, facilitate protein refolding, and promote the degradation of damaged proteins. Together, these functions protect cellular integrity and enable organisms to maintain homeostasis during periods of environmental stress. In the current study, transcript abundance of gill *hsp70* and *hsp90* increased significantly at high temperature in both male and female *P. reticulata*,

confirming that exposure to elevated temperature elicited a cellular stress response in the gill of these fish (Iwama et al., 1998; Feder and Hofmann, 1999; Deane et al., 2002). In contrast, no significant difference in *hsp70* or *hsp90* transcript abundance was observed between the medium- and low-temperature treatments, suggesting that the highest temperature alone exceeded the threshold necessary to activate the heat shock response. This confirms that the elevated experimental temperature treatment, at least, elicited a biologically relevant physiological stress response, providing confidence that any accompanying changes in TJ-associated transcripts were driven by thermal stress.

The upregulation of *hsp70* and *hsp90* observed in the present study are consistent with previous reports that have demonstrated increased transcript abundance of *hsp* in response to thermal stress across a range of teleost fishes. For example, heat stress significantly upregulated *hsp70* and *hsp90* transcripts in the liver of rainbow trout (*Oncorhynchus mykiss*) (Li et al., 2022), increased *hsp70* transcript abundance in the gill of goldfish (*Carassius auratus*) (Ngozichukwu and Rahman, 2026), and upregulated *hsp90α* and *hsp90β* transcripts in the liver of snow trout (*Schizothorax richardsonii*) (Barat et al., 2016). The observed response of gill *hsp70* and *hsp90* mRNA to low temperature in *P. reticulata* is not inconsistent with other studies because these molecular chaperones do not always respond to low temperatures. Instead, variable responses have been reported across species. For example, while some fishes upregulate HSPs during cold acclimation (Teigen et al., 2015), others exhibit little or no change in HSP abundance. In lake trout (*Salvelinus namaycush*), for example, cold exposure did not result in increased HSP abundance (Kelly et al., 2018). Similarly, Zarate and Bradley (2003) reported that cold stress did not alter gill *hsp30*, *hsp70*, or *hsp90* levels in Atlantic salmon (*Salmo salar*). The lack of a cold-induced *hsp70* and *hsp90* response in the present study is therefore consistent with previous observations in teleost fishes. Collectively, these findings indicate that cold-induced activation of the heat shock response is not universal and can vary among species, HSP families, tissues, and experimental conditions (Iwama et al., 2004; Reid et al., 2022).

4.3 Carcass moisture content

Carcass moisture content provides a measure of whole-body water balance and is commonly used as an indicator of salt and water balance in fishes (Al-Jandal and Wilson, 2011). In the present study, carcass moisture content did not differ significantly among temperature treatments, indicating that exposure to the experimental temperatures did not alter whole-body water content. Similarly, no significant sex-specific or interactive effect between temperature and sex was detected, suggesting that both male and female *P. reticulata* are able to maintain carcass moisture levels in the face of elevated and reduced water temperatures. The maintenance of consistent carcass moisture levels across temperature treatments is not unexpected, as stable muscle water content has similarly been reported in the Patagonian blennie (*Eleginops maclovinus*) following thermal acclimation and in Atlantic salmon (*Salmo salar*) during prolonged chilling (Rørvik et al., 2001; Oyarzún et al., 2018). Together, these findings suggest that whole-body water balance is relatively well-maintained in sub-optimal temperatures, despite other physiological responses that may occur in response to temperature stress.

4.3.1 Carcass chloride content

The maintenance of carcass chloride content observed in the present study is consistent with previous findings demonstrating that fishes can preserve ion homeostasis despite thermal stress. For example, Lahnsteiner and Dünser (2024) reported that rainbow trout exposed to near-freezing temperatures for 30 days maintained stable serum Cl^-, Na^+, and K^+ concentrations despite significant physiological stress, including increased *hsp90* expression, reduced growth, immunosuppression, and liver dysfunction. In addition, Lyytikäinen et al. (2002) found that Arctic charr (*Salvelinus alpinus*) maintained stable plasma Na^+, K^+, and Cl^- concentrations following four weeks of acclimation to temperatures between 10.3 and 18.1°C, indicating that ion homeostasis was preserved across a range of thermal conditions. Together, these findings suggest that compensatory osmoregulatory mechanisms can maintain whole-body ion balance even when thermal stress elicits substantial physiological and cellular responses (Lyytikäinen et al., 2002; Lahnsteiner and Dünser, 2024)

4.3.2 Gill Na^+/K^+-ATPase (NKA) activity

NKA is the primary ion-motive enzyme in the gill epithelium of fishes, and although it is ubiquitous in its cellular presence, it is particularly enriched in branchial ionocytes/MRCs and where it contributes to the generation of an electrochemical gradient that drives active ion acquisition across this tissue (McCormick, 2001). In the present study, males exhibited significantly lower gill NKA activity than females under low-temperature conditions, whereas no sex differences were observed at the medium (control) or high temperatures. This suggests that males and females may differ in their ionoregulatory response under low-temperature conditions. Although males exhibited significantly lower gill NKA activity than females under low temperature conditions, comparable sex-specific responses to thermal stress have not been widely reported. Nevertheless, sex-related differences in osmoregulatory performance have been documented in other teleosts. For example, mature female brook trout exhibited greater hypoosmoregulatory ability than mature males following seawater transfer, suggesting that sex and reproductive status can influence ionoregulatory capacity under salinity challenge (McCormick and Naiman, 1985).

4.4 TJ-associated genes

4.4.1 *cldn3* isoforms

In the mammalian TJ complex, CLDN3 is typically associated with enhancing epithelium barrier properties (Milatz et al., 2010; Günzel and Yu, 2013). This also holds true in fishes, where observations of increased gill Cldn3/*cldn3* isoform abundance has been shown to occur in circumstances where environmental change is known to cause a reduction in epithelium permeability (Chasiotis et al., 2012). Among the *cldn3* isoforms examined in this study, only *cldn3b* exhibited a significant temperature-dependent response, with transcript abundance increasing under high-temperature conditions in both males and females. In contrast, *cldn-3c* and *-3d* abundance remained unchanged across all temperature treatments. This differential response indicates that *cldn3* isoforms are not regulated uniformly during thermal stress.

This is consistent with how *cldn3* isoforms are found to selectively respond to environmental perturbation (Bagherie-Lachidan et al., 2008), which is in line with the idea of functional specialization among closely related Cldn family members (Günzel and Yu, 2013).

The selective upregulation of *cldn3b* observed in the present study suggests that in *P. reticulata*, this isoform may participate in the molecular remodeling of the gill epithelium during thermal stress by reinforcing the paracellular barrier. Although no studies have reported temperature-induced upregulation of *cldn3* in teleost fishes, similar responses have been observed in other vertebrates. For example, chronic heat exposure (35°C) significantly increased *cldn3* mRNA abundance in the cecum of broiler chickens, where the response was associated with heat-induced epithelial remodeling (Dung et al., 2024). Likewise, Pearce et al. (2013) reported increased CLDN3 protein expression in the ileum of heat-stressed pigs exposed to 35°C for 24 h, despite reduced intestinal barrier integrity, suggesting that increased CLDN3 expression formed part of the epithelial response to thermal stress. Together, these findings support the hypothesis that increased *cldn3b* expression represents a conserved molecular response that contributes to epithelial remodelling during heat stress. However, the specific function of *cldn3b* in *P. reticulata* remains to be determined, and further studies will be required to establish its role in the thermal response of the gill epithelium.

4.4.2 *cldn8* isoforms

Several *cldn8* isoforms exhibited significant temperature-dependent changes in transcript abundance. At high temperature, *cldn8a* and -*8d* mRNA abundance were increased in both male and female fish, indicating a conserved response to thermal stress. Because Cldn8 proteins are generally regarded as barrier-forming TJ proteins that reduce paracellular ion permeability (Günzel and Yu, 2013), the increased abundance of these isoforms in response to elevated temperature suggests that they promote or contribute to a reinforcement of the paracellular barrier through remodeling of the TJ complex. This interpretation is supported by the study of Kolosov and Kelly (2017), who demonstrated that Cldn8d functions as a barrier-forming protein in the rainbow trout gill. Specifically, cortisol-mediated upregulation of *cldn8d*/Cldn8d

increased transepithelial resistance (an overall measure of epithelial barrier function) and specifically reduced paracellular permeability. In addition, Na^+ and Cl^- flux across the trout gill epithelium (which represented a reduction in passive ion loss) also occurred following Cldn8d knockdown in a cultured trout gill epithelium model (Kolosov and Kelly, 2017).

In contrast, *cldn8b* and *-8c* exhibited variable patterns of abundance following experimental manipulation in this study, with responses influenced by both temperature and sex. Under high-temperature conditions, female fish generally exhibited higher transcript abundance than males, suggesting sex-specific regulation of these isoforms during thermal stress. Although the functional roles of *cldn8b* and *-8c* have not been established in *P. reticulata*, these differences may reflect variation in the molecular remodeling of the gill TJ complex between the sexes. As comparable sex-specific regulation of *cldn8* isoforms has not been widely reported in teleosts, the biological significance of this pattern remains unclear and requires further investigation.

4.4.3 *cldn10* and *cldn28* isoforms

In contrast to the temperature-responsive *cldn3* and *cldn8* isoforms, *cldn10* and *cldn28* isoforms exhibited relatively little variation across the experimental temperature treatments. Transcript abundance of all three *cldn28* isoforms (*cldn28a, -28b,* and *-28c*) remained unchanged in both males and females. Cldn28 isoforms are considered barrier-forming TJ proteins in fishes and are thought to contribute to restricting paracellular ion movement across the gill epithelium (Kolosov et al., 2013). Therefore, the absence of temperature-dependent changes in *cldn28* expression, despite significant responses among other predicted barrier-forming *cldns* such as *cldn3* and *cldn8*, suggests that temperature selectively regulates specific components of the branchial TJ complex rather than eliciting a uniform response across barrier-forming *cldns*.

A similar lack of temperature-dependent regulation was observed for *cldn10a, -10c, -10e,* and *-10f*. Unlike Cldn28, however, Cldn10 proteins are generally associated with the formation of selective

paracellular pores that facilitate cation movement (Chasiotis et al., 2012; Günzel and Yu, 2013). Although temperature had little influence on these isoforms, *cldn10b* and *cldn10d* exhibited consistent sex-specific differences, with transcript abundance being greater in females than males across temperature treatments. This finding suggests that sex, rather than temperature, may be an important factor regulating the expression of these particular *cldn10* isoforms.

The potential physiological significance of these sex-specific differences is particularly interesting given the proposed role of Cldn10 proteins in regulating paracellular cation permeability. In mummichog (*Fundulus heteroclitus*), *cldn10* isoforms are differentially regulated by environmental salinity, and changes in their expression are associated with the cation-selective properties of salt-secreting epithelia (Marshall et al., 2018). Accordingly, the consistently greater abundance of *cldn10b* and *cldn10d* in female *P. reticulata* raises the possibility that males and females differ in the constitutive regulation of paracellular cation movement across the gill epithelium. Such differences could contribute to sex-specific strategies for maintaining ion balance under changing environmental conditions. However, because transcript abundance alone does not establish functional permeability, direct measurements of gill ion permeability would be required to determine whether these sex-specific differences in *cldn10* expression translate into differences in paracellular cation movement.

4.4.4 *cldn15* isoforms

The transmembrane TJ protein Cldn15 contributes to the regulation of paracellular ion permeability and is classified as a pore-forming Cldn that facilitates the selective movement of cations including Na^+ and K^+ across epithelial tissues (Van Itallie and Anderson, 2006; Tamura et al., 2011; Kolosov et al., 2013). In the present study, modest but significant alterations in *cldn15* abundance were seen in response to temperature, but only when data were collapsed. Specifically, *cldn15a* transcript abundance increased at high temperature, whereas *cldn15b* transcript abundance increased at low temperature relative to the control treatment. No sex-specific alterations were observed in *cldn15* isoforms, and this suggests that the effects

of temperature were relatively subtle and may have been obscured by within-sex variability and sample size may have limited the statistical power to detect smaller effects.

The contrasting responses of *cldn15a* and *-15b* suggest that the two isoforms may be differentially regulated by temperature stress and potentially fulfil distinct physiological roles in the gill. Although the function of *cldn15* during thermal stress has not previously been investigated in teleost fishes, evidence from other vertebrates indicates that it is temperature responsive. Lassiter et al. (2024) reported that chronic cyclic heat stress significantly increased *CLDN15* transcript abundance in the lower duodenum and caecum of broiler chickens, alongside changes in other TJ proteins and genes involved in water homeostasis. While Lassiter et al. (2024) examined the avian intestine rather than the teleost gill, their results demonstrate that *CLDN15* can be regulated by elevated temperature across vertebrate epithelia.

4.4.5 *cldn30* and *cldn31* isoforms

In the gill of *P. reticulata*, several *cldn30* isoforms exhibited temperature-dependent changes in transcript abundance, although the response varied among individual isoforms. Specifically, *cldn30a*, *-30d*, and the barrier-forming *cldn31* (see Kolosov et al., 2017) displayed significant temperature-dependent variation, indicating that these TJ-associated elements are responsive to temperature stress and may contribute to remodeling of the gill TJ complex when thermal conditions are sub-optimal. In contrast, *cldn30b* exhibited a more subtle response, with significant temperature-dependent differences detected only when data were pooled across sexes, suggesting that variability between males and females may have reduced the statistical power to detect treatment effects in the sex-specific analyses.

Unlike the other *cldn30* isoforms, *cldn30c* remained unchanged across all temperature treatments, indicating that its abundance is stable under the experimental conditions. This finding suggests that not all members of the *cldn30* family are involved in the molecular response to temperature stress, further supporting the concept of functional specialization among closely related *cldn* isoforms (Chasiotis et al., 2012; Kolosov et al., 2013).

4.4.6 *ocln* isoforms

Ocln is an integral transmembrane protein of the TJ complex that contributes to the maintenance of epithelial barrier integrity through interactions with scaffolding proteins and the actin cytoskeleton (Furuse et al., 1993). In the present study, transcript abundance of *ocln-a* and *ocln-b* remained unchanged across all temperature treatments, and no significant differences were detected between males and females. These findings indicate that neither temperature stress nor sex influence *ocln* in the gills of *P. reticulata* under the conditions examined. However, temperature-dependent regulation of gill *ocln* has been reported in other teleosts. In spotted seabass (*Lateolabrax maculatus*), Cheng et al. (2021) found that gill *ocln* mRNA expression was significantly lower in fish maintained at 33°C than in those maintained at 27°C. Thus, the absence of an *ocln* response in *P. reticulata* contrasts with the response observed in spotted seabass and suggests that regulation of *ocln* during temperature stress may be species-specific or dependent on the severity and duration of exposure. However, Varasteh et al. (2015) reported that *ocln* mRNA abundance remained unchanged in the intestines of heat-stressed chickens, suggesting that *ocln* transcription may remain relatively stable during temperature stress in some vertebrates (Varasteh et al., 2015).

In contrast, several mammalian studies have reported increased Ocln protein abundance following heat stress. Pearce et al. (2013) found that acute heat stress increased Ocln protein abundance in the porcine ileum despite a concurrent reduction in epithelial barrier integrity. The authors proposed that this increase represented a compensatory response to heat-induced barrier dysfunction rather than an improvement in barrier function and further suggested that much of the increased Ocln accumulated within the cytosol rather than being incorporated into the TJ complex. Similarly, Dokladny et al. (2006) demonstrated that heat exposure increased Ocln protein expression and promoted its localization to the TJ complex in cultured human intestinal epithelial cells. Inhibition of HSP expression prevented this compensatory increase in Ocln and disrupted its junctional localization, suggesting that HSPs contribute to the regulation of Ocln during heat stress. Collectively, these studies suggest that across vertebrate clades, Ocln responds to temperature stress differently, but more studies are needed to reinforce this idea.

4.4.7 *zo-1*

ZO-1 is a cytoplasmic scaffolding protein that links transmembrane TJ proteins to the actin cytoskeleton, thereby contributing to TJ assembly and epithelial barrier integrity (Furuse et al., 1994; Fanning et al., 1998). In the present study, *zo-1* transcript abundance increased in response to increased temperature stress, suggesting that the remodeling of *P. reticulata* gill TJ complex induced by thermal challenge requires the participation of more than transmembrane elements. There is one other study that reports on the effect of temperature on gill zo-1 transcript abundance, where it was found that exposure of spotted seabass (*Lateolabrax maculatus*) to 33°C significantly decreased gill *ZO-1* expression relative to fish maintained at 27°C, whereas dietary arginine partially restored *ZO-1* expression, suggesting a protective effect against heat-induced disruption of the gill barrier (Cheng et al. 2021). This difference may reflect species-specific variation or differences in osmoregulatory strategy between FW and marine teleosts. FW fishes experience continual passive ion loss and water influx across the gills and therefore rely on a relatively tight branchial epithelium to minimise diffusive ion loss while facilitating active ion uptake. In contrast, marine teleosts experience continual osmotic water loss and passive salt gain and regulate branchial permeability to facilitate salt secretion while conserving body water (Evans et al., 2005; Marshall et al., 2018). These contrasting physiological demands may influence how the gill TJ complex responds to thermal stress. However, comparative studies examining the effects of temperature on *zo-1* regulation in FW and marine teleosts are lacking, and further research is required to determine whether environmental salinity contributes to these contrasting responses.

Studies of other environmental stressors likewise demonstrate that *ZO-1* expression is responsive to environmental challenge, although the direction of the response appears to depend on the nature of the stressor. For example, copper exposure significantly decreased *zo-1* transcript abundance in the gills of juvenile grass carp, whereas dietary arginine prevented this reduction, suggesting maintenance of epithelial barrier integrity (Wang et al., 2015). In contrast, combined exposure of rainbow trout gill epithelial cells to ZnO and TiO_2 nanoparticles increased *zo-1* transcript abundance, which was interpreted as a compensatory

response to cellular stress rather than evidence of improved barrier function (Beghin et al., 2024). Collectively, these findings demonstrate that *zo-1* is responsive to environmental stress, but the direction of the response varies among species, stressors, and exposure conditions. The increase in *zo-1* transcript abundance observed in the present study contrasts with the decreases reported following heat exposure in spotted seabass and copper exposure in grass carp but resembles the increase observed following combined nanoparticle exposure (Wang et al., 2015; Cheng et al., 2021; Beghin et al., 2024).

4.4.8 *mr*

The selective remodelling of the TJ complex observed in the present study may be mediated, at least in part, through corticosteroid signalling. Temperature stress can activate the hypothalamic-pituitary-interrenal axis in teleost fishes, resulting in the release of cortisol, a major corticosteroid involved in coordinating the physiological stress response (Mommsen et al., 1999; Kültz, 2005). In addition to its roles in metabolism and osmoregulation, cortisol acts through glucocorticoid and mineralocorticoid receptors to regulate ionoregulatory processes and epithelial barrier function (McCormick, 2001; Evans et al., 2005). Consistent with a potential role for this pathway, mineralocorticoid receptor (*mr*) transcript abundance was significantly elevated under high temperature stress in the present study. Because cortisol can activate the teleost MR (Sturm et al., 2005), increased *mr* transcript abundance may alter the responsiveness of the gill epithelium to corticosteroid signalling during thermal challenge. However, increased receptor transcript abundance does not necessarily correspond to greater receptor protein abundance or activity.

Evidence from rainbow trout gill epithelia supports a role for MR in regulating the paracellular barrier. Cortisol increases transepithelial resistance, reduces paracellular permeability, and alters the abundance of several TJ-associated proteins (Chasiotis et al., 2010; Kelly and Chasiotis, 2011). More specifically, *mr* knockdown reduced transepithelial resistance, increased paracellular permeability, and decreased transcript abundance of several barrier-forming *cldns*, including *cldn6, -8d, -23a,* and *-28b* (Kolosov and Kelly, 2019). These findings demonstrate that MR contributes to gill epithelial barrier function and influences the composition of the branchial TJ complex.

Importantly, corticosteroid regulation of the TJ appears to be selective. In cultured rainbow trout gill epithelia, cortisol increased the abundance of the barrier-forming proteins Cldn8d and Cldn28b (Kolosov and Kelly, 2019). However, following *mr* knockdown, the cortisol-induced increase in Cldn28b protein abundance was lost, whereas Cldn8d remained responsive to cortisol. In addition, *mr* knockdown decreased the transcript abundance of *cldn6, cldn8d, cldn23a,* and *cldn28b*, while increasing *cldn20a* (Kolosov and Kelly, 2019). These findings suggest that MR contributes to the regulation of specific Cldns rather than uniformly regulating the branchial TJ complex. Similarly, *cldn31* knockdown increased epithelial permeability and diminished, but did not eliminate, cortisol-induced epithelial tightening in cultured trout gill epithelia (Kolosov et al., 2017). Furthermore, *cldn31* knockdown abolished the cortisol-induced increase in Cldn8d abundance, indicating that corticosteroid-mediated changes in gill barrier properties may involve coordinated responses among individual Cldns.

These findings provide context for the temperature-dependent changes in *mr* and TJ-associated transcripts observed in the present study. In particular, the temperature responsiveness of *cldn8* isoforms is notable because Cldn8d has been identified as a cortisol-responsive, barrier-forming Cldn in rainbow trout gill epithelium (Kolosov and Kelly, 2017). Cortisol selectively increased *cldn8d* transcript abundance in rainbow trout, whereas *cldn8b* and *cldn8c* were unaffected, further demonstrating that corticosteroid responsiveness can differ among closely related Cldn isoforms (Kolosov and Kelly, 2017). In contrast, *cldn28* isoforms remained unchanged in the present study despite evidence that Cldn28b can be regulated through MR in rainbow trout (Kolosov and Kelly, 2019). Thus, the increase in *mr* transcript abundance observed in *P. reticulata* did not coincide with a uniform response among TJ components associated with corticosteroid signalling. Instead, these findings may reflect selective regulation of individual TJ proteins during temperature stress.

In addition to its established role in regulating TJ composition and epithelial barrier function, *mr* transcript abundance itself can be influenced by environmental temperature in teleost fishes. Temperature-dependent changes in *mr* transcript abundance have been reported in Atlantic salmon, where exposure to

elevated temperatures altered telencephalic *mr* expression in a temperature- and exposure-dependent manner (Tang et al., 2022). Although this response was observed in the telencephalon rather than the gill, they demonstrate that *mr* transcript abundance is responsive to temperature stress and provide evidence that temperature-dependent regulation of *mr* may occur across tissues in teleost fishes.

4.5 Sex-specific differences

The consistent sex-specific differences observed in the relative abundance of several encoding TJ proteins suggest that sex may be an important biological factor influencing the molecular response to temperature stress. Although the mechanisms underlying these differences remain unclear, previous studies have demonstrated that vertebrates can exhibit distinct sex-specific physiological and molecular responses to elevated temperatures. For example, Wang et al. (2020) reported that acute heat stress elicited markedly different transcriptomic responses in the gonads of male and female Chinese tongue sole (*Cynoglossus semilaevis*), while Yanar et al. (2023) identified modest but significant sex-specific differences in thermal tolerance among several poeciliid species. One potential explanation for these differences is hormonal regulation, as Cldn expression is known to be influenced by sex hormones, including estradiol, progesterone, testosterone, and gonadotropins, in other vertebrates (Kozieł et al., 2020). Although similar mechanisms have not yet been demonstrated in teleost gills, the present findings suggest that sex may influence the regulation of TJ genes during temperature stress.

Importantly, almost all previous studies investigating the effects of temperature on epithelial barrier function and osmoregulation in teleost fishes have not explicitly considered sex as a biological variable (Metz et al., 2003; Cheng et al., 2021; Li et al., 2022; Li et al., 2023; Lahnsteiner and Dünser, 2024). Consequently, sex-specific patterns in the molecular response to temperature stress may have been overlooked or masked by combining data from males and females, not taking the sex of experimental animals into consideration or using immature fish. While some of the expression patterns observed in the present study differ from those reported in earlier investigations, these discrepancies may reflect the meaningful biological variability introduced by sex rather than inconsistencies among studies. Opposing

molecular responses between males and females may become obscured when data from both sexes are pooled, potentially leading to an oversimplified understanding of paracellular regulation during temperature stress.

Collectively, the present findings suggest that, in addition to the well-established role of transcellular transport in the temperature response of fishes, regulation of the paracellular pathway may also contribute to sex-specific responses to temperature stress. Although the functional significance of these transcriptional differences remains to be determined, the present findings highlight the importance of incorporating sex as a biological variable in future studies investigating gill epithelium barrier function, osmoregulation, and temperature adaptation in teleost fishes.

5. CONCLUSION

The present study investigated the effects of temperature on osmoregulatory function in *Poecilia reticulata*, with a particular focus on the regulation of gill TJ-associated genes and sex-specific responses. It was hypothesised that barrier-forming TJ proteins in the FW fish gill would exhibit sex-specific alterations in abundance at sub-optimal temperatures as the gill epithelium reorganized to mitigate paracellular ion loss. This hypothesis was supported, as sub-optimal temperatures altered the transcript abundance of several barrier-forming TJ genes, with a number of these responses differing between males and females. Importantly, the response was selective rather than universal, indicating that thermal stress regulates specific components of the TJ complex rather than producing a uniform response across all TJ-associated genes. Elevated temperature also elicited a clear cellular stress response, as demonstrated by increased *hsp70* and *hsp90* transcript abundance, consistent with the established role of HSPs in the cellular response of fishes to thermal stress (Iwama et al., 1998; Feder and Hofmann, 1999).

Several barrier-forming *cldns*, including members of the *cldn3, cldn8, cldn30,* and *cldn31* families, exhibited increased transcript abundance under thermal stress. Given the importance of the gill TJ complex in controlling paracellular permeability and limiting passive ion loss in FW fishes, these responses are consistent with a potential adjustment of the epithelial barrier to maintain ionoregulatory homeostasis (Chasiotis et al., 2012). In contrast, several *cldn10* and *cldn28* isoforms, as well as both *ocln* isoforms, remained unchanged with temperature, further demonstrating the selective nature of the TJ response. Sex-specific patterns were also evident, most notably for *cldn10b* and *cldn10d*, which exhibited consistently greater transcript abundance in females than males. Because Cldn10 proteins have been implicated in the formation and maintenance of cation-selective paracellular pores in teleost gills (Marshall et al., 2018), these differences raise the possibility that males and females differ in the regulation of paracellular cation movement.

These molecular responses occurred despite relatively stable whole-animal measures of water and ion balance across temperature treatments. Gill NKA activity, however, revealed a distinct sex-specific

response to low temperature. In males, NKA activity was significantly lower at low temperature than at the medium (control) temperature and was also significantly lower than that of females under the same low-temperature conditions. In contrast, female NKA activity did not differ among temperature treatments. Thus, the relative stability of whole-animal water and ion balance does not necessarily indicate an absence of underlying ionoregulatory adjustments. Rather, the male-specific reduction in NKA activity indicates a response in a major component of transcellular ion transport, while changes in TJ-associated transcripts demonstrate concurrent regulation of the paracellular pathway.

Together, the NKA and TJ responses highlight sex as an important factor influencing gill ionoregulation in *P. reticulata*. The male-specific reduction in NKA activity at low temperature, combined with sex differences in the abundance of select TJ transcripts, suggests that males and females may differ in how transcellular and paracellular pathways contribute to maintaining ion balance under thermal challenge. Importantly, some sex-specific patterns were no longer apparent when males and females were pooled such that only temperature was considered. Accounting for sex therefore revealed biologically relevant responses that would otherwise have been overlooked.

Collectively, this study demonstrates that the gill ionoregulatory response to temperature in *P. reticulata* is selective and can be influenced by sex. Rather than inducing a generalized response across the gill epithelium, temperature stress altered specific TJ-associated transcripts, while low temperature additionally reduced NKA activity in males. These findings support the hypothesis that gill TJ components exhibit sex-specific alterations under sub-optimal temperatures and suggest that regulation of the paracellular pathway may contribute to the maintenance of ion balance in sub-optimal temperatures.

In natural environments, FW temperatures fluctuate daily and seasonally. However, as an initial investigation, the present study used static temperature treatments to isolate the effects of sustained low- and high-temperature exposure under controlled conditions. Future studies should therefore examine

fluctuating temperature regimes that more closely reflect natural environmental conditions (Reeve et al., 2014; Morash et al., 2021).

Future studies should determine whether these transcriptional responses translate into changes in TJ protein abundance, localization, epithelial permeability, and paracellular ion movement, as changes in *cldn* transcript abundance do not necessarily establish corresponding changes in TJ protein abundance or epithelial function (Kolosov et al., 2013; Kolosov and Kelly, 2016). In particular, determining whether the greater *cldn10b* and *cldn10d* transcript abundance in females corresponds to differences in paracellular cation permeability would help clarify the physiological significance of these sex-specific patterns, given that Cldn10 proteins contribute to cation-selective paracellular pathways in fish gills (Marshall et al., 2018). Measurements of whole-body and/or plasma Na^+ concentrations would help determine whether temperature- and sex-dependent changes in TJ gene expression and NKA activity ultimately influence Na^+ balance, particularly because FW fishes must actively acquire Na^+ while limiting its diffusive loss across the gill epithelium (Evans et al., 2005; Zimmer and Perry, 2022). Imaging of TJ proteins within the gill epithelium could further establish whether changes in transcript abundance correspond to altered protein abundance and localization at the TJ. As well, future studies should measure circulating cortisol concentrations to determine whether cortisol-mediated MR activation contributes to temperature-dependent TJ regulation. Given the observed increase in *mr* transcript abundance and evidence that MR regulates gill epithelial permeability and barrier-forming Cldns (Kolosov and Kelly, 2019), this would help clarify whether MR signalling contributes to gill epithelial remodelling in the context of temperature stress.

Beyond providing insight into how the gill epithelium TJ complex responds to temperature stress, these findings may have broader ecological implications for fishes inhabiting FW ecosystems experiencing increasingly fluctuating and extreme temperatures (Maberly et al., 2020; Capon et al., 2021). The sex-specific responses observed here suggest that males and females may differ in their physiological sensitivity to temperature stress, which, if associated with differences in survival or fitness, could potentially skew

population sex ratios and influence population stability under changing temperature conditions (Wedekind et al., 2013; Geffroy and Wedekind, 2020).

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