A ROLE FOR FENUGREEK IN ALTERING THE OSMOREGULATORY CAPACITY IN RAINBOW TROUT (*Oncorhynchus mykiss*)

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Abstract

Fenugreek (*Trigonella foenum-graecum*) is a botanical galactogogue that has been shown to increase milk production in mammals. Although the precise mechanism(s) of fenugreek action are unknown, it is thought to act on the prolactin pathway. Prolactin, a principal hormone involved in milk production in mammals, is classically considered to be a freshwater-adapting hormone in teleost fishes. If fenugreek promotes prolactin synthesis in vertebrates, then in fishes fenugreek has the potential to decrease systemic perturbation associated with environmental change in a hypoosmotic environment and/or exposure to hypoosmotic conditions. To test this notion, rainbow trout (*Onchorhynchus mykiss)* were fed varying doses of fenugreek and end points of ionoregulatory ability were examined. A single dose was then selected for use in a hypoosmotic stress experiment. Dietary fenugreek did not disrupt systemic end points of salt and water balance, however the number of mitochondria-rich cells in the gill increased. Gill V-type H^+-ATPase activity decreased with dietary fenugreek. Transcript abundance of prolactin receptor in the gill and hypothalamus and corticosteroid receptors in the gill decreased. Transcript abundance of the freshwater subunit of Na^+-K^+-ATPase increased in the 50μg/g dose of fenugreek. High doses of fenugreek resulted in increased transcript abundance of the tight junction genes *claudin-7*, and *-30*, and decreased abundance of *cldn*-33b. While it was demonstrated that fenugreek can impact salt and water balance in the rainbow trout, the hypothesis that these changes would be brought about via prolactin was not supported.

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Table of contents

Abstract……………………………………………………………………………ii

Acknowledgements…………………………………………………………….iii

List of tables……………...……………………………………………………....vi

List of figures…………………………………………………………………vii

List of abbreviations………………………………………………………......ix

1.0 Introduction…………………………………………………………………....1

1.1 The challenges of life in FW.......................…………………………......1

1.2 Hormones involved in FW fish osmoregulation......................................3

1.3 Vertebrate epithelia..................................……………………………....4

1.4 The role of the gill in FW fish osmoregulation…...................................…..6

1.5 Botanicals…………………………..…………………….……………………...8

1.6 Hypothesis…………………………………………………………………11

2.0 Methods…………………………………………………………………………..13

2.1 Experimental animals……………………………………...…………......13

2.2 Preparation and administration of experimental diets..…………………13

2.3 Tissue sampling……………………..…………………………………....14

2.4 Muscle moisture content and serum [Cl^-] and [Na^+] analyses…………15

2.5 Gill enzyme activities……………………………………………………..…...16

2.6 RNA extraction, cDNA synthesis and qRT-PCR analyses……….....……17

2.7 Primer design……………………………………………………………...19

2.8 Scanning electron microscopy and gill morphometrics…………………23

2.9 Immunohistochemistry………………………………………..……………...23

2.10 Statistical analyses……………………………………………………....25

3.0 Results……………………………………………………………………...27

3.1 Dose-dependent effect of dietary fenugreek……………………………………27

3.1.1 Muscle moisture content, serum [Cl^-] and [Na^+]...............................27

3.1.2 Gill MRC morphometrics...27

3.1.3 Gill NKA & VA transcript abundance and activity.............................27

3.1.4 TJ protein transcript abundance in the gill..28

3.1.5 *prl, prrp* and hormone receptor transcript abundance in the gill, pituitary, and hypothalamus tissue……………………………………………28

3.2 Effect of dietary fenugreek and IPW……………………………………………..38

3.2.1 Muscle moisture content, and serum [Cl^-], [Na^+]...............................38

3.2.2 Gill MRC morphometrics..38

3.2.3 Gill NKA & VA transcript abundance and activity..............................38

3.2.4 TJ protein transcript abundance in the gill...39

3.2.5 *prl, prrp* and hormone receptor transcript abundance in the gill, pituitary, and hypothalamus tissue……………………………………………..39

4.0 Discussion……………………………………………………………………51

4.1Overview………………………………………………………………51

4.2 Dose-dependent effect of dietary fenugreek...51

4.3 Effect of dietary fenugreek and IPW..58

4.4 Conclusions and perspectives……………………………………………..65

5.0 References……………………………………………………………………67

6.0 Appendix: Skin……………………………………………………………..82

List of tables

Table 1: Primer set information………………………………………………….......21

Table 2: Primer set information………………………………………………….......22

Dose-dependent effect of dietary fenugreek

Table 3: Muscle moisture, and serum [Cl^-] and [Na^+] analyses 29

Effect of dietary fenugreek and IPW

Table 4: Muscle moisture, and serum [Cl^-] and [Na^+] analyses 41

List of figures

Figure 1: FW osmoregulation..2

Figure 2: The vertebrate tight junction complex......……………………………...6

Dose-dependent effect of dietary fenugreek

Figure 3: Gill MRC surface morphometrics......…......……………..……………...30

Figure 4: Gill Na^{+}-K^{+}-ATPase (NKA) immunoreactivity and NKA-immunoreactive cell numbers...……………………………..31

Figure 5: Gill NKA and H^{+-}ATPase subunit transcript abundance and activity.....32

Figure 6: Gill TJ protein transcript abundance...33

Figure 7: Gill claudin transcript abundance..34

Figure 8: Gill hormone receptor transcript abundance.......................................35

Figure 9: Hypothalamus hormone receptor transcript abundance.......………36

Figure 10: Pituitary *prl* and hypothalamus *prrp* transcript abundance.................37

Effect of dietary fenugreek and IPW

Figure 11: Gill surface morphometrics...................……………..…………....42

Figure 12: Gill Na^{+}-K^{+}-ATPase (NKA) immunoreactivity and NKA-immunoreactive cell numbers..…………………………...43

Figure 13: Gill NKA and H^{+-}ATPase subunit transcript abundance and activity...44

Figure 14: Gill claudin transcript abundance..45

Figure 15: Gill TJ protein transcript abundance...46

Figure 16: Gill hormone receptor transcript abundance.....................................47

Figure 17: Hypothalamus hormone receptor transcript abundance....................48

Figure 18: Pituitary *prl* and hypothalamus *prrp* transcript abundance……………49

Appendix

Figure 19: Dose-response skin claudin qRT-PCR..84

Figure 20: Dose-response skin tight junction and scaffolding qRT-PCR………...85

List of abbreviations

ADP – antibody dilution buffer

ANOVA – analysis of variance

cDNA – complementary deoxyribonucleic acid

CDS – coding sequence

Cgn – cingulin

Cldn – claudin

DAPI – 4’,6’-diamidino-2-phenylindole

dd-H_2O – double-distilled water

FITC – fluorescein-isothiocyanate

FW – freshwater

IPW – ion-poor water

ISME – ion-selective microelectrode

LRDs – Licorice root derivatives

MRC – mitochondria rich cell

mRNA – messenger ribonucleic acid

NADH – nicotinamide adenine dinucleotide

NKA – Na^+, K^+ ATPase

Ocln – occluding

PBS – phosphate buffered saline

PF – Kodak Photo-Flo 200

Prl – prolactin

PrlR – prolactin receptor

PrRP – prolactin-releasing peptide

PVC – pavement cell

qRT-PCR – quantitative real time polymerase chain reaction

SEM – scanning electron microscopy

SE – standard error of the mean

SW – saltwater

TJ – tight junction

Tric - tricellulin

TX – Triton X-100

VA – vacuolar-type H^+-ATPase

ZO-1 – zonula occludens-1

1.0 INTRODUCTION

1.1 The challenges of life in freshwater

As with any habitat, there are many challenges associated with life in an aquatic environment. Due at least in part to the high specific heat of water, most teleosts conform to environmental temperatures. Therefore organisms must be able to cope with fluctuations in temperature. Water contains relatively little oxygen compared to air, although freshwater (FW) has a higher capacity to dissolve oxygen than saltwater (SW) (Gilbert et al. 1968). Thus, efficient oxygen acquisition is a requirement in aquatic media. However a problem faced uniquely in FW environments is constant immersion in a dilute medium. In a hypoosmotic medium, organisms face a passive obligatory efflux of ions to the environment and water influx down their respective diffusive gradients (Fig. 1). To maintain salt and water balance, animals must continuously counteract these fluxes.

Salt and water balance in fish is a task undertaken by many organs including the kidney, intestine, skin and gill (Farrell 2011). The passive influx of water and efflux of ions is offset by the production of copious amounts of dilute urine, as well as the active uptake of ions across the gill. The kidney and intestine are responsible for ion absorption, as well as urine excretion and decreased water absorption respectively. The skin acts primarily as a barrier to prevent diffusive fluxes of ions and water between the organism and the environment.

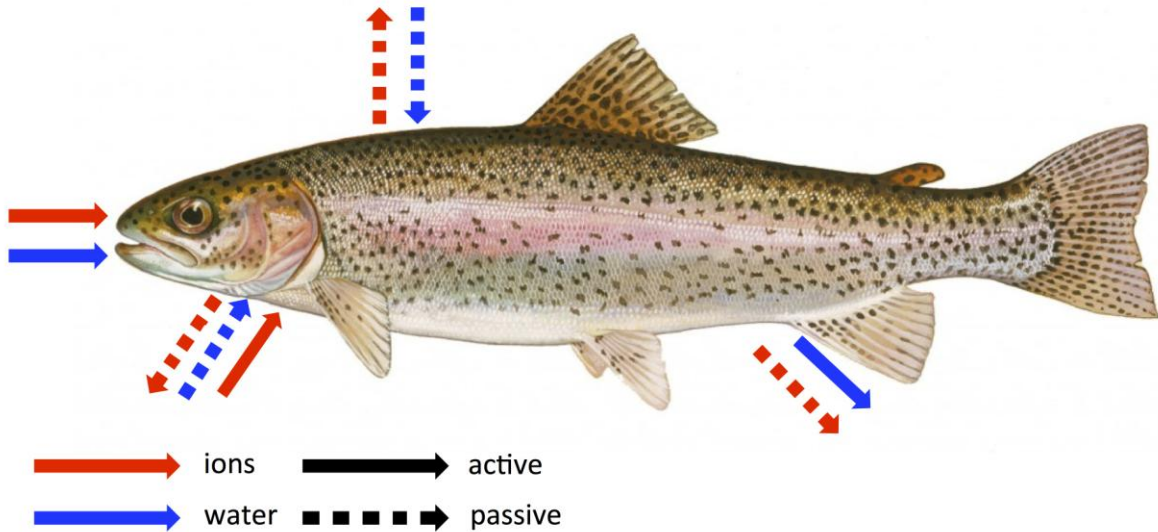


Figure 1: A generalized model of the movement of water (blue) and ions (red) in the freshwater teleost fish. Unbroken arrows represent active processes, while dotted arrows represent passive processes governed by concentration gradients. Ions are actively acquired across the gill, and via food. Copious amounts of dilute urine are produced by the kidney and excreted.

Movement between FW and SW, as seen in euryhaline fish such as the rainbow trout, requires modifications to osmoregulatory organs in order to cope with the gradient reversals of ions and water. For instance, the SW-acclimated gill secretes ions instead of actively uptaking them, as is the case in the FW-acclimated gill. The SW-acclimated gill is also considered to be composed of a "leakier" epithelium as opposed to the FW-gill (Bartels and Potter 1991). As the central connection between physiological response and environmental perturbations, the neuroendocrine system is important in bringing about the osmoregulatory response.

1.2 Hormones involved in freshwater osmoregulation

In euryhaline fish, hormonal control of FW acclimation has been linked primarily by two hormones: prolactin (Prl) and cortisol. Prl has an abundance of documented functions in vertebrates including osmoregulation, reproduction, growth, effects on ectodermal structures, and steroid synergism (Nicoll and Bern 1972, Bole-Feysot 1998). In teleost fish, it has a central role in maintaining salt and water balance, particularly in a FW environment (Pickford and Phillips 1959, McCormick and Bradshaw 2006, Sakamoto and McCormick 2006). In euryhaline fish Prl gene expression, synthesis, secretion, and circulating levels all increase following transfer from SW to FW (Manzon 2002). Prl is known to alter the "SW-type" mitochondria-rich cell (MRC), or ionocyte, as well as induce the reappearance of the "FW-type" MRCs of the gill (Pisam et al. 1993). Prl receptors (PrlR) in the gill have been localized primarily to MRCs in multiple teleost species, indicating that these are the main targets of Prl action in the gill (Lee et al. 2006, Weng et al. 1997, Santos et al. 2001). Prl is shown to affect the morphology, number, size and distribution of MRCs in FW-acclimated fish (McCormick 1995), and those acclimated to a hypoosmotic environment (Kelly et al. 1999). Prl also decreases the permeability of the gill epithelium to ions (Kelly and Wood 2002, Hirano 1986). Although a solid link between this observation and the physiology of the tight junction (TJ) complex has yet to be established, Claudin (Cldn)-28a and -28b have exhibited increased transcript abundance with elevated Prl levels and FW transfer respectively (Tipsmark et al. 2009, Sandbichler et al. 2011). While Prl is known to bring about a FW acclimation

response, it is not the only hormone involved in the regulation of salt and water balance in FW fishes.

Although classically considered to be a SW-adapting hormone, cortisol also has a role in FW adaptation. Cortisol has been shown to promote ion uptake and reduce paracellular permeability of the gill epithelia (McCormick 2001, Zhou et al. 2003). In some cases, cortisol has been observed to interact with Prl to induce a FW-acclimation response (Zhou et al. 2003). Cortisol, a glucocorticoid, was long thought to be the only major producer of corticosteroid actions in teleosts (Kelly and Chasiotis 2011, Takahashi and Sakamoto 2013), however the recently discovered mineralocorticoid receptor (MR) can be bound by 11-deoxycorticosterone in an agonistic fashion. Blocking of the MR results in a reduction of the MRC proliferation associated with FW acclimation (Sloman et al. 2001).

1.3 Vertebrate epithelia

Vertebrate epithelia are tissues where adjoining cells share common polarity (*i.e.* apical and basal sides) and associate with the basement membrane on the basal side (Tyler 2003). Although diverse, a common characteristic of epithelia is that they serve to separate compartments. As such, epithelia serve to form a barrier for the uncontrolled passage of solutes and water (Gunzel and Fromm 2012). Paracellular permeability of epithelial cells is regulated at the apicolateral junction by the TJ complex, a belt-like reticular network of proteins that form a selective paracellular barrier (Furuse 2010, González-Mariscal et al. 2003). The vertebrate TJ is composed of tetraspan transmembrane proteins

including Cldns and TJ-associated MARVEL proteins (TAMPs), including occludin (Ocln) at the bicellular contacts, as well as tricellular TJ proteins, including tricellulin (Tric) (Raleigh et al. 2010) (Fig. 2). Interaction between the extracellular domains of transmembrane TJ proteins allows the modulation of the selective passage through the paracellular pathway. Transmembrane TJ proteins attach to the cytoskeleton via scaffolding TJ proteins such as zonula occludens-1 (ZO-1) and cingulin (Cgn) (Itoh et al. 1999).

In addition to the paracellular pathway, movement of solutes and water across epithelia can occur through transcellular means (Farrel 2011). Transport through the cell is regulated via primary and secondary active transport (as well as passive diffusion) by integral membrane ion channels and transporters. As would be expected, perturbations in environmental osmolarity, such as those experienced by euryhaline fish, result in modulations to the regulators of both the paracellular and transcellular pathways (Clelland et al. 2010, Bui and Kelly 2014, Evans 2005, Marshall and Grosell 2006).

As a result of a whole genome duplication event from ancestral teleosts (Allendorf and Thorgaard 1984), these organisms have an expansive Cldn family with at least 33 genes described so far (Tipsmark et al. 2008, Kolosov et al. 2013, Kolosov et al. 2014). The Cldn family consists of tetraspanning membrane proteins that can form homo- or, in some cases, heterotypic interactions with Cldns across the paracellular gap (Van Itallie and Anderson 2013, Gunzel and Yu 2012, Gunzel and Fromm 2013).

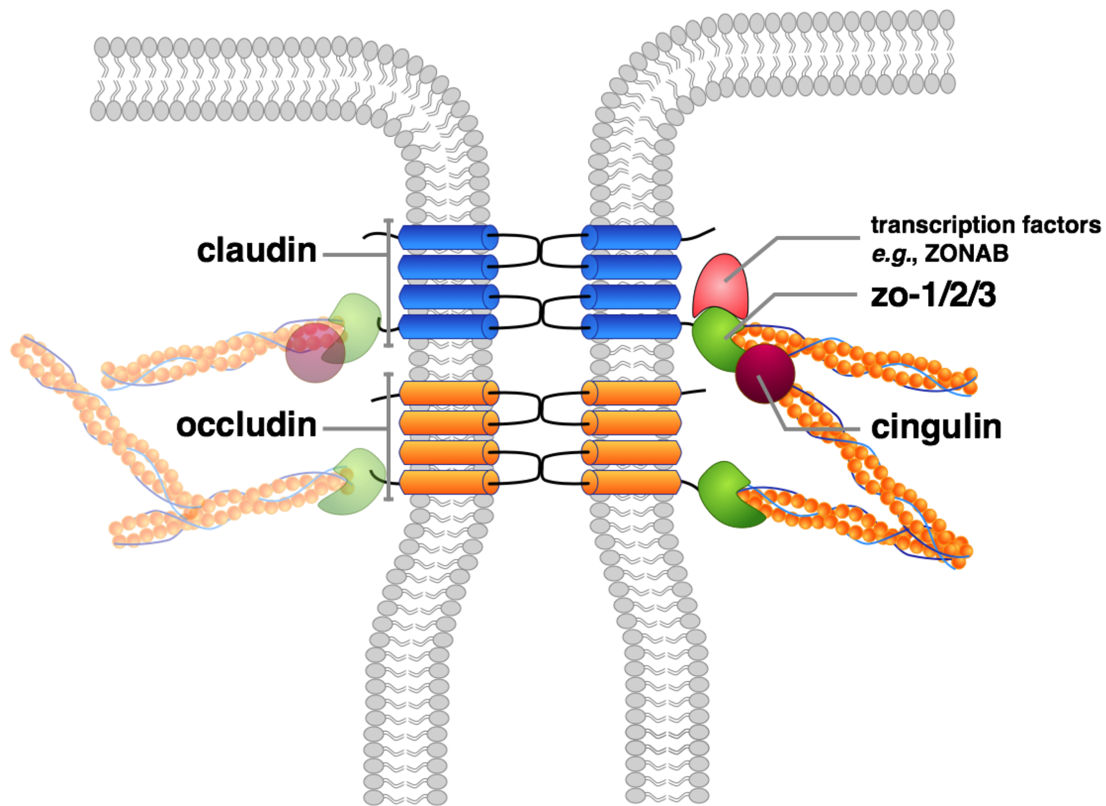


Figure 2: A simplified model of the tight junction (TJ) complex at the junction between epithelial cells. Transmembrane proteins, such as claudins (blue) and occludin (orange), span the membrane four times. These proteins regulate paracellular permeability through interaction with their counterparts across the junction. The transmembrane TJ proteins are connected to cytoskeletal elements, *e.g.* actin, via ZO-1/2/3 (green and orange), which also interact with transcription factors (pink). Cingulin (maroon and orange) interacts with zo-1/2/3 and cytoskeletal elements. Both cingulin and ZO-1/2/3 are known to regulate gene expression and cell proliferation (Chasiotis 2012b).

1.4 The role of the gill in freshwater fish osmoregulation

By virtue of its role in respiration, acid-base balance, salt and water balance, and excretion, the gill is optimized to facilitate exchange between the internal milieu and the external environment (Evans 2005). Due to the extensive surface area in contact with the environment, high perfusion rate, and short

diffusion distance of the gill (Palzenberger and Pohla 1992), any changes that are a result of environmental perturbations will manifest in the gill first.

The surface of the gill is composed of two primary cell types: pavement cells (PVCs) and MRCs, which are sometimes referred to as mitochondria-rich cells or chloride cells in the literature. PVCs comprise ~90% of the epithelial surface area, while MRCs comprise ~10%. PVCs are considered to be primarily passive barriers with respect to osmoregulation (Evans et al. 2005). MRCs are rich in mitochondria, and are the main sites of active ion uptake in the FW fish gill. The energy produced by mitochondria is used to drive the activity of ion transporters such as Na^{+}-K^{+}-ATPase (NKA) and V-type H^{+}-ATPase (VA) (Evans et al. 2005), which aid in maintaining ion homeostasis by facilitating ion uptake. In FW, MRCs are typically localized on the primary filament along the base of the lamellae, and in the interlamellar region. There are at least two distinct types of MRC found in rainbow trout (Hiroi et al. 2005, Dymoska et al. 2012), the α-type MRC is found in both FW and SW, and the β-type, which is found exclusively in FW-acclimated fish (Pisam et al. 1987, Galvez et al. 2001).

1.5 Botanicals

Botanicals have been used for centuries to treat a wide variety of ailments, and for their health-promoting properties (Bent and Ko 2004, Craig 1998). With a few exceptions, the use of herbal remedies is considered to be safe, and their use in the United States has been steadily increasing (Bent and Ko 2004). As the demand for ethical (organic, hormone-free, etc.) practices in food production continues to rise, the use of botanical supplements (*i.e.* nutraceuticals) may

become an accepted means of improving the health and condition of animals raised for food. While the use of herbal medicine in humans is prevalent, the effects of botanicals on fish physiology are mostly unexplored.

Recently, Chen et al. (2015) investigated the effects of licorice root derivatives (LRDs) on salt and water balance in the rainbow trout. The LRDs glycyrrhizic acid and glycyrrhetinic acid can bind to corticosteroid receptors (Ulmann et al. 1975), and are used to ameliorate the effects of stress. As previously mentioned, cortisol in fish is responsible for restructuring the gill epithelia upon experiencing environmental perturbations in salinity. Chen et al. (2015) determined that with the feeding of LRDs to rainbow trout, there was no disruption to physiological end points of salt and water balance in normal environmental conditions. However some differences were noted in biochemical and molecular end points of gill osmoregulatory function.

Botanical substances that promote milk production (*i.e.* galactogogues) are often prescribed as a “natural” means to stimulate lactation in pre-term births in midwivery. The primary outcome of treatment observed with various galactogogues was a change in serum Prl (Mortel and Mehta 2013). Prl, among its many physiological roles, is a lactation-stimulating hormone in mammals. A botanical galactogogue that is known to affect Prl levels could prompt a change in the osmoregulatory capacity of a fish, particularly in FW.

Fenugreek, *Trigonella foenum-graecum* L, is an annual legume. As one of the oldest known herbal medicines, fenugreek is used in traditional medicine for its lactogenic, anti-diabetic, anti-microbial, anti-cancer and hypocholesterolaemic

properties (Acharya et al. 2006a, Acharya et al. 2006b). It is commonly prescribed in Ayurvedic and Chinese medicine, and in midwifery as a galactagogue. There is abundant anecdotal evidence for the efficacy of fenugreek as a lactation stimulant, with documented successes reported as early as 1945 (Gabay et al. 2002). Experimentally, fenugreek has been shown to enhance milk production in lactating mammals (Al-Shaikh et al. 1999, Alamer and Basiouni 2005). This effect seems to be associated with Prl, as fenugreek treatment has been reported to increase Prl levels using multiple doses (Samia et al. 2012). However, in another instance, fenugreek had no effect at all, where only a single dosage was investigated (Reeder et al. 2013). Nevertheless there is an absence of congruity; it is difficult to draw conclusions on the effect of fenugreek specifically on Prl due to the relative scarcity and inconsistency of experimental research. For example, in one case, ground seeds are used, in another, capsules from a pharmacist, in another, unlisted. However, the preponderance of evidence suggests that fenugreek not only has an effect on Prl, but also increases its circulating levels. It is well known that Prl plays a central role in milk production and lactation (Ostrom 1990, Buhimschi 2004), so it seems very plausible that the effects of fenugreek would be brought about via Prl.

In addition, there is also evidence supporting the role of fenugreek extract as a phytoestrogen. A component of fenugreek extract can competitively bind to estrogen receptors (ER) in MCF-1 cells, and it was shown to act in an agonistic fashion (Sreeja et al. 2010). Mice that were fed fenugreek extract exhibited

elevated circulating estrogen levels in their serum (Modaresi et al. 2012). 17β-estradiol has been shown to stimulate Prl synthesis and release in rainbow trout and sea bream (Barry and Grau 1986, Williams and Wigham 1994, Brinca et al. 2003). Conversely, estrogen treatment has been shown to inhibit SW acclimation in brown trout (Madsen and Korsgaard 1991). While fenugreek extract contains many different components; the phytoestrogenic activity of fenugreek is consistent with diosgenin.

Diosgenin, a major medicinally active component of fenugreek seeds, is a steroidal sapogenin, *i.e.* it contains a steroid framework (Marker and Krueger 1940). Estrogenic activity has been demonstrated in the unaltered diosgenin, while no effect on progesterone levels was seen (Aradhana and Rao 1992). Diosgenin can be chemically converted into progesterone, and it is thought that this conversion could take place endogenously in some organisms, however it is a complex process, and has not been demonstrated in vivo (Marker and Krueger 1940, Au et al. 2004). In mammals, progesterone has been shown to inhibit the estrogen-induced increase in serum [Prl], however on its own, progesterone can increase Prl secretion (Chen and Meites 1970).

Thus the phytoestrogenic properties of fenugreek extract could activate estrogen receptors, and/or elevate estrogen levels, and has the potential to act on Prl to alter osmoregulatory capacity of in rainbow trout.

1.6 Hypothesis

A botanical that does have an effect on salt and water balance may in fact enhance the osmoregulatory capacity of the fish. For instance, a “tightening” of the gill epithelia might result in less ion leakage, and thus a reduced need to counter loss with active ion uptake. Or, a botanical could have a priming effect, increasing the ability of the fish to cope with potential fluxes in environmental salinity. A botanical galactogogues that is known to affect Prl levels could prompt a change in the osmoregulatory capacity of a fish, particularly in FW.

To the best of my knowledge, no study has considered the idea that a botanical galactogogues could impact salt and water balance in fishes by acting on Prl. Therefore treatment with fenugreek in teleost fishes might be expected to alter salt and water balance and/or end points associated with ionoregulatory homeostasis. If fenugreek does indeed alter the osmoregulatory capacity of rainbow trout, then it has potential as a dietary supplement to improve the ability to cope with changes in ion composition of the water, as well as reducing physiological stress from environmental insults in rainbow trout.

To explore this idea further, two experiments were run. First, a dose-response experiment was run to determine the effect of fenugreek on end points of salt and water balance, and with a further emphasis on gill morphology and biochemistry in FW-acclimated rainbow trout. Second, the effect of fenugreek with regard to hypoosmotic stress will be tested using fenugreek-fed rainbow trout in the presence of environmental insults *i.e.*, IPW. Lastly, the potential modes of action will be examined. The rainbow trout was used as a model

organism as the mechanisms governing salt and water balance has been well documented in this euryhaline fish. Juvenile fish were used as they were observed to cope with IPW stress better than adults.

2.0 MATERIALS AND METHODS

2.1 Experimental animals

Rainbow trout (*Oncorhynchus mykiss;* ~65g) were obtained from Humber Springs Trout Club and Hatchery, Orangeville, ON, Canada. Following transfer to the laboratory, fish were held in 200 L opaque polyethylene aquaria (n = 24 fish per tank) supplied with flow-through, dechlorinated City of Toronto tap water (approximate composition in μM: Na^+ 590; Cl^- 920; Ca^{2+} 900; K^+ 50; pH 7.4). Water was aerated. During acclimation to laboratory conditions, fish were fed ad libitum once daily with commercial trout pellets (Martin Mills Profishent, Elmira, ON, Canada). Water temperature was around 13 ± 1°C and fish were held under a constant photoperiod (14 h light:10 h dark).

2.2 Preparation and administration of experimental diets

Fenugreek (Fenugreek absolute, natural) was obtained from Sigma-Aldrich, Oakville, ON, Canada. Fenugreek was administered to fishes via the diet using techniques previously adopted for the oral administration of hormones to fishes (Bernier et al. 2004). Diets containing various doses of fenugreek (5, 50, and 500μg fenugreek/g diet) were prepared by dissolving fenugreek in absolute ethanol and spraying the solution evenly on to commercial trout pellets (Martin Mills, Elmira, ON, Canada). Excess ethanol vessel was evaporated at room temperature, and diets were stored at -20°C until use. The control diet was prepared in an identical fashion, however only absolute ethanol was applied. Fish were fed experimental diets over a 14-day period at 3% body weight at the same time once daily.

For the ion-poor water (IPW) challenge, the experiment was set up in an identical fashion to the dose-response experiment using the 0 and 50 μg/g diet. After 14 days of feeding regime, twenty fish per group were sampled. The remaining fish were fed and the tank water and water supply was quickly switched to IPW (approximate composition in μM: Na^{+} 20; Cl^{-} 40; Ca^{2+} 2; K^{+} 0.4; pH 7.4). Fish were then sampled after 24 hours.

2.3 Tissue sampling

Rainbow trout were quickly net captured and anesthetized in 1.0 $g{\bullet}L^{-1}$ tricaine methasulfonate (MS-222; Syndel Laboratories Ltd, Canada) prior to tissue sampling. Blood was then collected from the caudal vein using 500μl syringes (BD Falcon TM; BD Biosciences, Mississauga, ON, Canada), and fish were killed by spinal transection. Blood was allowed to clot for 30 min at room temperature, and then centrifuged (4°C, 10600 g, 10 min) to obtain serum. The serum was then aliquoted and stored at -80°C until further analyses.

The first gill arch from the left branchial chamber was collected for messenger RNA (mRNA) analyses. Gill arches from the right branchial chamber were collected for enzyme activity assays, scanning electron microscopy (SEM) and immunohistochemistry. A section of skin anterior, and dorsal to the lateral line was removed for mRNA analyses. The pituitary and hypothalamus were removed for mRNA analyses. All samples for mRNA extraction and enzyme activity assays were flash frozen immediately upon collection and stored at -80°C until further analyses. A section of epaxial muscle tissue (dorsal to the lateral line) was collected for determination of muscle moisture content.

Fish husbandry, animal experiments and tissue collection took place in accordance with an approved York University Animal Care Protocol and conformed to Canadian Council on Animal Care guidelines.

2.4 Muscle moisture content, and serum [Cl^-] and [Na^+] analyses

Muscle moisture content was determined gravimetrically by drying a known weight of fresh muscle tissue at 60°C until it reached a constant weight, and the relative difference in mass was the moisture content of the muscle.

Serum [Cl^-] was determined colorimetrically in accordance with methods outlined by Zall et al (1956). This assay measures the formation of ferric thiocyanate, which relies on the dissolution of mercuric thiocyanate via the formation of mercuric chloride. Thus the amount of ferric thiocyanate is directly proportional to chloride content. Samples were promptly read at 480 nm using a Multiskan^{TM} Spectrum microplate reader (Thermo Electron Corp, Nepean, ON, Canada).

Serum [Na^+] was determined using an ion-selective microelectrode (ISME) as described in Jonusaite et al. (2011). To construct liquid membrane ISME, borosilicate glass capillaries (TW150-4; WPI, Sarasota, FL, USA) were heated and pulled to a tip diameter of ~5 µm using a P-97 Flaming-Brown micropipette puller (Sutter Instruments Co., Novato, USA) and salinized with vapours of N,N-dimethyltrimethylsilylamine (TMS) at 300^oC for 45 min. ISMEs were backfilled with 100mM NaCl and tips were front-loaded via capillary action with a short column length (250–300 µm) of Na^+X ionophore, which was chosen for its high-selectivity for Na^+ (Messerli et al. 2008). ISME tips were dipped in a

solution of polyvinylchloride in tetrahydrofuran (both from Fluka, Buchs, Switzerland) to prevent the ionophore from leaking. ISMEs were calibrated in 10μl droplets of 30 mM NaCl/270 mM LiCl and 300 mM NaCl, ISME slopes (mV) for a tenfold change in ion concentration were 55.4 +/- 0.4 for both experiments (mean ± SE, n). The circuit for voltage measurements was completed using a reference electrode constructed from borosilicate glass capillaries (IB200F-4, WPI, Sarasota, FL, USA) backfilled with 500 mmol KCl. The electrodes were connected through an ML 165 pH Amp to a PowerLab 4/30 (AD Instruments Inc., Colorado Springs, CO, USA) data acquisition system and the voltage readings were recorded and analyzed using LabChart 6 Pro software (AD Instruments Inc.,). Serum Na^+ activities were calculated using the following equation, as described by Donini et al. (2007): $a^h = a^c \times 10^{(\Delta V/S)}$ where a^h is the serum [Na^+], a^c is the [Na^+] in one of the calibration solutions, ΔV is the difference in voltage readings between the serum and the calibration solution, and S is the slope of the electrode measured in response to a tenfold difference in [Na^+] between the calibration solutions.

2.5 Gill enzyme activities

Gill NKA and VA enzyme activity was determined according to methods outlined by McCormick (1993). Tissue was homogenized (3200g, 4°C, 10 min) on ice in a 1:15 weight:volume SEID (250 mM Sucrose, 10mM Na^+EDTA, 50mM imidazole, 12mM deoxycholic acid). The homogenate was centrifuged to collect the supernatant. An assay mixture containing lactate dehydrogenase, pyruvate kinase, phosphoenolpyruvate, NADH and imidazole (pH 7.5) was prepared on

the day of the assay. Two additional mixtures were prepared by adding either ouabain to inhibit NKA activity or bafilomycin A1 (BioShop Canada Inc., Burlington, ON, Canada) to inhibit VA activity to the original assay mixture. A salt solution containing 189 mM NaCl, 10.5 mM MgCl,, 42 mM KCl, and 50 mM imidazole (pH 7.5) was prepared. The assay mixtures were combined with the salt solution (3:1) and used for the determination of enzyme activity in the 10µl of homogenate supernatant.

Enzyme activity was calculated by measuring the linear rate of NADH consumption at 340nm for 30 min using a Multiskan^{TM} Spectrum microplate reader. NKA and VA activities were determined by calculating the difference between nicotinamide adenine dinucleotide (NADH) consumption in assay solutions. Enzyme activity was standardized to protein concentration in supernatant, which was determined using Bradford Reagent (Sigma Life Science, MO., USA). Enzyme activities are expressed in µmol adenosine diphosphate•mg protein^{-1}•hour^{-1}.

2.6 RNA extraction, cDNA synthesis and qRT-PCR analyses

Total RNA was extracted from gill, skin, pituitary and hypothalamus tissue using TRIzol reagent (Life Technologies, Burlington, ON, Canada) according to the manufacturer instructions. Tissue was homogenized in TRIzol reagent, and then incubated at room temperature for five minutes. Chloroform was added to each sample and mixed vigorously. To separate the RNA phase from DNA and protein, the samples were centrifuged (4°C, 12000 g, 15 min), and the upper phase was collected. The sample was incubated with 2-propanol before

centrifuging (4°C, 12000 g, 10 min) to precipitate the RNA. 2-propanol was removed before washing with 75% ethanol. The sample was centrifuged (4°C, 7500 g, 5 min), before removing the ethanol and adding autoclaved diethylpyrocarbonate (DEPC)-treated water. Total RNA yield from each sample was quantified using a Multiskan™ Spectrum Photometer.

Prior to complementary deoxyribonucleic acid (cDNA) synthesis, mRNA samples were treated with DNase I (Amplification Grade; Life Technologies) before inactivating the DNases for 10 min at 65°C. mRNA samples were hybridized by adding Oligo(dT)$_{12-18}$ and dNTP (Invitrogen Canada Inc.) and incubating for 5 min at 65°C. Subsequently, SuperScript™ III Reverse Transcriptase, 5x first strand buffer and DTT (Invitrogen Canada Inc.) were added before incubating at 50°C for 60 minutes, then inactivating the reaction at 70°C for 15 minutes.

Quantitative real-time PCR (qRT-PCR) was conducted using iQ SYBR® Green (Bio-Rad Laboratories Inc. Canada), gene specific primers (Table 1 and 2), and autoclaved double-distilled water (dd-H_2O). First the reaction vessel and contents were heated at 95°C for 15 minutes to activate the polymerase and denature the cDNA. It was then cycled for 15 sec at 95°C to denature the cDNA, then at the annealing temperature (annealing temperatures for each primer set listed in Table 1) for 60 sec for primer annealing extension of the amplicon. The fluourescence of the sample was measured. This cycle was repeated 40 times. A melting curve analysis was conducted to assess the dissociation characteristics of the product. qRT-PCR was used to quantify mRNA

abundance of genes encoding TJ proteins and transcellular ion transport proteins, as well as genes encoding for Prl, PrlR, Prl releasing peptide (PrRP), and the estrogen receptor subunits, ER, ERβ1, and ERβ2. Transcellular ion transport genes studied were the NKA subunits α1a and α1b (*nka-α1a, and -α1b*), and VA. qRT-PCR results were normalized to either β-actin or elongation factor-1α using ΔΔC(t) method as described by Livak and Schmittgen (2001).

2.7 Primer design

Primers for rainbow trout *prl*, *prrp*, *prlr*, *er*, *erβ1*, *erβ2*, and *cldn-28a* were designed as follows. The coding sequence (CDS) for the gene of interest in rainbow trout was obtained from the National Centre for Biotechnology Information website. If the gene had not been sequenced in rainbow trout (*Oncorhynchus mykiss*), the sequence was obtained in the Atlantic salmon (*Salmo salar*). The CDS was then used to search for similar sequences in rainbow trout using the Basic Local Alignment Search Tool (BLAST), as was the case for Cldn-28a. In the case of PrRP, there were no rainbow trout sequences in the database that were similar to the *S. salar* CDS, so the primer set was designed against the *S. salar* CDS. Once the CDS was obtained, potential primer sets were generated using Primer3 (Rozen and Skaletsky 2000, Koressaar and Remm 2007, Untergrasser et al. 2012). Primers were tested for their propensity to bind to themselves or the other primer in the set using OligoAnalyzer 3.1. Primer sets were then tested for their specificity for the gene of interest using BLAST. Suitable primer sets were then ordered from (Life Technologies, Canada Inc.). The primer sets were then used to run PCR. The PCR product was run in

agarose gel electrophoresis to ensure that a single amplicon was amplified. The product was also sequenced to ensure that the amplicon matched the gene of interest. Primers were designed *for prl, prrp, prlr, cldn-28a, er, erβ1* and *erβ2* which are included unsourced alongside the other primer sets used in qRT-PCR analyses in Table 1.

Table 1: Primer sets, corresponding amplicon size, annealing temperature, and GenBank accession numbers for claudin (*cldn*)*-28a,* prolactin (*prl*), prolactin-releasing peptide (*prrp*), endocrine receptors (*prlr*, *er*, *er*β1*, er*β2), transcellular transport proteins (*nka α1a, nka α1b, vaβ*), tight junction proteins (*tric*, *ocln*, *cgn*, *zo-1*), and reference genes (*actβ*, *ef-1α*)

Gene	Primer sequence (5' to 3')	Amplicon size (bp)	Annealing temperature (°C)	Accession number
prl	F: GTCACACCTCCTCACTCCAGAC R: CAGTCCATCTCCCAGGCTC	220	61	NM_001124733
prrp	F: GAATAACCTGGCACGCGGAA R: GAGCAGCAACAGAATCGTGA	180	60	DQ021886.1
prlr	F: CTAATCCTGCCTGTGGTTGC R: CCCGCTGTATGGTAGTCTGG	205	61	NM_001124599.1
gr1 [a]	F: GGACTGAAACACAGCAAGGAC R: GCAATACTCGCCTCCAACAG	335	59	NM_001124730
gr2 [a]	F: AGAACACGTCTGCCATGC R: CTGGAGAAAGCGGAGGTAG	346	57	NM_001124482
mr [a]	F: TGTGTCTGGGTAATGGTAGC R: CGTTGTTGTTGTTCTCTTGG	369	56	AY495584
er	F: AGACCTCGTCCACACCCTTA R: ACCGCACAGTACCTCGTCTC	162	60	NM_001124349
*er*β1	F: GTGACTCTGCGACCCTTCTG R: GTGCCCGATGTCAGTGTATG	157	61	NM_001124753
*er*β2	F: CGTCTGTCTGAAAGCCATGA R: GATGACTGCTGCTGGAATGA	170	60	NM_001124570
nka α1a [b]	F: AGAAAGCCAAGGAGAAGATG R: AGCCCGAACCGAGGATAGAC	133	56	NM_001124461.1
nka α1b [b]	F: AGCAAGGGAGAAGAAGGACA R: GAGGAGGGGTCAGGGTG	176	59	NM_001124460.1
*va*β [b]	F: CAACCCTCAGTGCCGTATC R: GAAGAAGCGAGCAGTTTCC	259	60	NM_001124570
tric [c]	F: GTCACATCCCCAAACCAGTC R: GTCCAGCTCGTCAAACTTCC	170	60	KC603902
ocln [d]	F: CAGCCCAGTTCCTCCAGTAG R: GCTCATCCAGCTCTCTGTCC	341	58	GQ476574
cgn [e]	F: CTGGAGGAGAGGCTACACAG R: CTTCACACGCAGGGACAG	156	56	BK008767
zo-1 [a]	F: AAGGAAGGTCTGGAGGAAGG R: CAGCTTGCCGTTGTAGAGG	291	60	HQ656020
actb [a]	F: GGACTTTGAGCAGGAGATGG R: GACGGAGTATTTACGCTCTGG	355	58	NM_001124753
ef-1α [e]	F: GGCAAGTCAACCACCACAG R: GATACCACGCTCCCTCTCAG	170	60	NM_001124570

[a]Kelly and Chasiotis 2011; [b]Chen et al. 2015; [c]Kolosov and Kelly 2013; [d]Chasiotis et al. 2010; [e]Kolosov et al. 2014.

Table 2: Primer sets, corresponding amplicon size, annealing temperature, and GenBank accession numbers for the claudin family of tight junction proteins.

Gene	Primer sequence (5' to 3')	Amplicon size (bp)	Annealing temperature (°C)	Accession number
cldn-1[b]	F: GAGGACCAGGAGAAGAAGG R: AGCCCCAACCTACGAAC	182	60	BK008768
cldn-3a[a]	F: TGGATCATTGCCATCGTGTC R: GCCTCGTCCTCAATACAGTTGG	285	60	BK007964
cldn-5a[b]	F: CTCACCGTCATCTCGTGTG R: CATCCAGCAGAGGGGAAC	171	59	BK008769
cldn-6[b]	F: TGAAACCACGGGACAGATG R: TGAAACCACGGGACAGATG	245	60	KF445436
cldn-7[a]	F: CGTCCTGCTGATTGGATCTC R: CAAACGTACTCCTTGCTGCTG	261	61	BK007965
cldn-8b[b]	F: ACGACTCCCTCCTGGCTCT R: GAGACCCATCCGATGTAGA	185	56	BK008770
cldn-8c[b]	F: GCTTGATGTGCTGCTCTC R: CCCAGAGGTCAGGAGGA	201	60	BK008771
cldn-8d[a]	F: GCAGTGTAAAGTGTACGACTCTCTG R: CACGAGGAACAGGCATCC	200	60	BK007966
cldn-10c[b]	F: CCTGGTCTGCTCTACAATGC R: CCCGAAGAATCCCAAAATAA	223	58	BK008772
cldn-10d[b]	F: CTGCTTTGTGTCGTGTCTGG R: AGCGAAGAACCCAAGGATG	253	60	BK008773
cldn-10e[b]	F: CTGTCACCAACTGCCAAGAC R: CCAGAAAGCCACTGATGATG	216	54	KF445437
cldn-12[a]	F: CTTCATCATCGCCTTCATCTC R: GAGCCAAACAGTAGCCCAGTAG	255	60	BK007967
cldn-13[b]	F: AGCGGCACTCTGGACAA R: CGGAAACCACACCTCTCC	231	59	BK008774
cldn-23a[b]	F: ATCCTAAACCTCACAGCGACA R: CGGTCTTTCCAGCACCTTAC	270	60	BK008775
cldn-27b[b]	F: GCCAACATCGTAACAGGACA R: CCAGAAGAGCACCAATGAGC	283	60	BK008776
cldn-28a	F: GACCTGCGTTGACTCTCCTC R: GGGTTACACAGTTCATCCACAA	245	60	BX320641.3
cldn-28b[a]	F: CTTTCATCGGAGCCAACATC R: CAGACAGGGACCAGAACCAG	310	60	EU921670
cldn-29a[b]	F: CTTTCATCGGCAATAACATC R: CAGCAATGGAGAGCAGG	201	60	BK008777
cldn-30[a]	F: CGGCGAGAACATAATCACAG R: GGGATGAGACACAGGATGC	297	59	BK007968
cldn-31[a]	F: TCGGCAACAACATCGTGAC R: CGTCCAGCAGATAGGAACCAG	311	61	BK007969
cldn-32a[a]	F: ATTGTGTGCTGTGCCATCC R: AGACACCAACAGAGCGATCC	321	60	BK007970
cldn-33b[b]	F: GTCCACAGACCTCTTGCTCAC R: TCCTGCCACCTTCATAATCC	268	60	BK008778

[a]Kelly and Chasiotis 2011; [b]Kolosov et al. 2014.

2.8 Scanning electron microscopy and gill morphometrics

SEM samples were prepared according to methods described in (Chasiotis et al., 2012a). Dehydrated gill filaments were mounted onto SEM stubs and sputter-coated (Hummer VI Au/Pd 40/60, Anatech USA; 2 min). Samples were then examined using a Hitachi S-520 SEM (Hitachi High-Technologies Canada, Toronto, Canada) attached to a Quartz PCI Version 6 image capture system (Quartz Imaging, Vancouver, Canada). Images were taken on a relatively flat surface on the afferent edge of the proximal end of the primary filament.

Analysis of images was conducted using ImageJ (National Institutes of Health, Java 1.6.0_65 (32-bit)). Gill morphometrics were determined according to methods previously described (Chasiotis et al., 2012a). The borders of MRCs were traced in ImageJ to calculate the surface area. Average MRC size was calculated using only cells which fell entirely within the image. Cell surface fractional area was calculated by dividing the total surface area of all MRCs by the total surface area of the image. Number of MRCs was calculated by dividing the total surface area of MRCs per image by the average size.

2.9 Immunohistochemistry

Gill samples used for immunohistochemistry were prepared according to methods outlined by Chasiotis and Kelly (2008). Rainbow trout gill tissues were fixed in Bouin's solution for 4 hours at room temperature. Fixed gill samples were then washed and stored in 70% ethanol at 4^0C until further analyses. Tissues were dehydrated in an ascending ethanol series (70-100%), cleared with xylene,

then infiltrated and embedded with Paraplast Plus Tissue Embedding Medium (Oxford Worldwide, LLC, Memphis, TN, USA). 3 μm sections were cut using a Leica RM 2125RT manual rotary microtome (Leica Microsystems Inc., Richmond Hill, ON, Canada). Sections were then collected on glass slides and dried overnight. Paraffin was removed using xylene, and then the samples were rehydrated to water *via* a descending series of ethanol rinses (100–50%), and subjected to heat-induced epitope retrieval (HIER). HIER was accomplished by immersing slides in a jar of sodium citrate buffer (10mM, pH 6.0) and heating the solution and slides in a microwave oven for 4 min. The jar with slides was cooled for 20 minutes, reheated for two minutes, and cooled for another 15 minutes. Slides were then washed three times with phosphate-buffered saline (PBS, pH 7.4). The slides were then quenched for 30min in 3% H_2O_2 in PBS. The H_2O_2 was rinsed from the slides with distilled water, the slides were then successively washed with 0.4% Kodak Photo-Flo 200 in PBS (PF/PBS, 10min), 0.05% Triton X-100 in PBS (TX/PBS, 10min), and 10% antibody dilution buffer (ADB; 10% goat serum, 3% BSA and 0.05% Triton X-100 in PBS) in PBS (ADB/PBS, 10 min). Slides were incubated overnight at room temperature with mouse monoclonal anti- NKA α-subunit antibody (α5, 1:10 in ADB; Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Sections were successively washed with PF/PBS, TX/PBS and ADB/PBS (10min each), then incubated with fluorescein-isothiocyanate (FITC)-labelled goat anti-mouse antibody (1:500 in ADB; Jackson ImmunoResearch Laboratories, Inc.) for 1h at 37°C. Slides were then successively washed with PF/PBS, TX/PBS and PF/PBS (10min each) and

rinsed 3 times with 0.4% PF in distilled water (PF/dH2O, 1min each). Slides were air dried for 1h and mounted with ProLong® Gold Antifade Mountant with 4',6'-diamidino-2-phenylindole (DAPI)(Life Technologies, Burlington, ON, Canada).

Fluorescence images were captured using an Olympus IX71 inverted microscope (Olympus Canada, Richmond Hill, ON, Canada) equipped with an X-CITE 120XL fluorescent Illuminator (X-CITE, Mississauga, ON, Canada). Images were taken of sections that transected the central sinus and the lamellae at the maximal length. NKA-rich cells in these sections were counted and expressed per mm of primary filament in order to standardize the measurements across images.

2.10 Statistical analyses

Data are expressed as mean values +/- the standard error of the mean (SE), where n represents the number of fish, or the number of pooled samples per treatment. For the dose-response experiment, significant differences were detected on SigmaPlot using a one-way analysis of variance (ANOVA) followed by a Holm-Sidak multiple comparisons procedure. For the IPW exposure, significant differences were detected using a two-way ANOVA. All significant results were followed with a Holm-Šídák multiple comparison test, unless otherwise stated (SigmaPlot Build 11.0.0.77, Systat Software Inc., www.sigmaplot.com). If necessary, data were normalized using a logarithmic transformation.

3.0 RESULTS

3.1 Dose-dependent effect of fenugreek

3.1.1 Dose-dependent effect of fenugreek on muscle moisture content, and serum [Cl^-], or [Na^+]

Diets containing varying levels of fenugreek did not significantly alter muscle moisture content, serum [Cl-], or [Na^+] in rainbow trout (Table 3).

3.1.2 Dose-dependent effect of fenugreek on gill MRC morphometrics

In examining the surface morphology of the primary filament, it was found that fenugreek-fed fish displayed a decrease in the number of MRCs exposed on the gill surface in both the 50 and 500 μg/g doses compared to the control. However, fenugreek did not alter the average size of MRCs, or the total MRC cell surface fractional area (Fig. 3).

The number of MRCs per unit length of primary filament did not differ significantly between treatments as determined by IHC and fluorescent imaging (Fig. 4).

3.1.3 Dose-dependent effect of fenugreek on NKA & VA transcript abundance and activity in the gill

Of the ion transporter subunits, *nka-α1a* exhibited a difference between the 5 μg/g and 50 μg/g fenugreek treatments ($p=0.01$), *nka-α1b* and *vaβ* did not change (Fig. 5 A-C).

Fenugreek treatment did not significantly alter NKA or VA activity in the gill (Fig. 5 D,E).

3.1.4 Dose-dependent effect of fenugreek on TJ protein transcript abundance in the gill

No differences were seen in *ocln* and *tric* (Fig. 6 A,B). Similarly, abundance of the scaffolding protein transcripts *cgn* and *zo-1* (Fig. 6 C,D) did not change with fenugreek treatment.

Of the 21 members of the Cldn family of tight junction proteins investigated following treatment with fenugreek, significant differences were observed in the levels of transcripts encoding 7 Cldns (Fig. 7). These differences were exhibited in one, some or all fenugreek treatments. Transcript abundance of *cldn-3a cldn-5a*, and *cldn-33b* all decreased with fenugreek treatment. Transcript encoding *cldn-7*, *cldn-27b*, *cldn-30*, and *cldn-31* all exhibited an increase in abundance with fenugreek treatment.

3.1.5 Dose-dependent effect of fenugreek on *prl, prrp,* and hormone receptor transcript abundance in gill, pituitary, and hypothalamus tissue

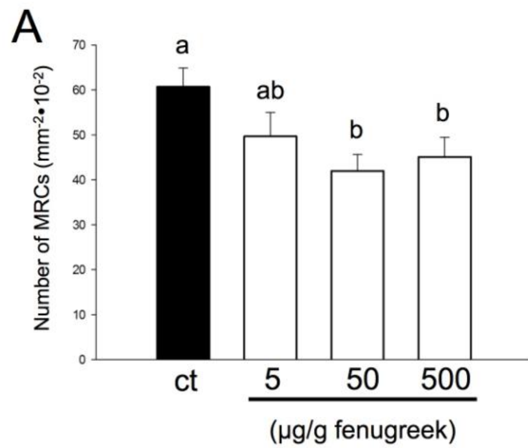
In the gill, significant differences were seen in the hormone receptors *prlr* ($p=0.019$), *gr1* ($p=0.013$), *mr* ($p=0.005$) (Fig. 8). No changes were observed in *gr2*, or the estrogen receptors *er*, *erβ1*, and *erβ2.*

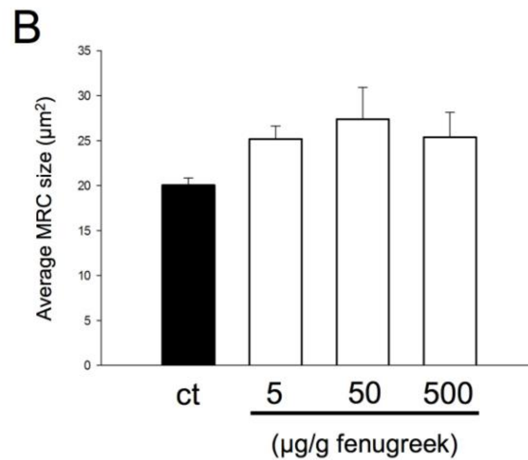
In the hypothalamus, *prlr* transcripts exhibited a significant decrease in the 50 µg/g group ($p=0.019$), however the estrogen receptors *er*, *erβ1*, and *erβ2* remained unchanged (Fig. 9). No differences were seen in *prl* or *prrp* transcript abundance in the pituitary and hypothalamus respectively (Fig. 10).

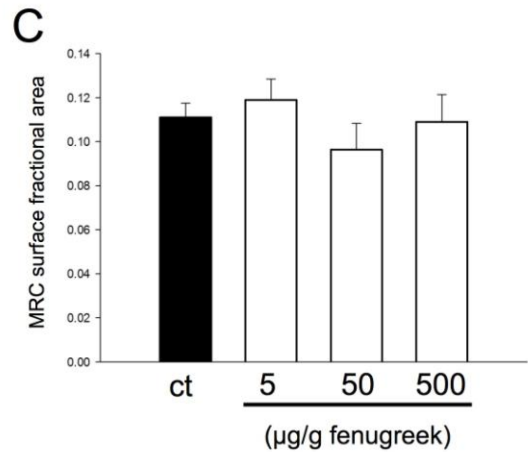
Table 3: Effect of various doses of dietary fenugreek (0, 5, 50, 500 μg fenugreek/g diet) on muscle moisture and serum [Na^+] and [Cl^-] in freshwater rainbow trout.

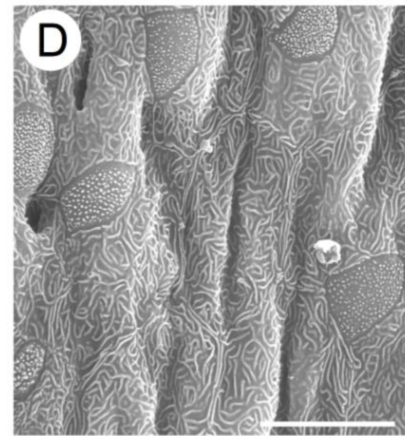
μg/g diet	Muscle Moisture (%)	Serum [Cl^-] (mM)	Serum [Na^+] (mM)
0	78.4 ± 0.24	118.7 ± 3.9	156.8 ± 3.6
5	78.0 ± 0.53	117.1 ± 3.7	152.0 ± 5.2
50	77.8 ± 0.37	115.8 ± 3.3	159.5 ± 6.5
500	77.9 ± 0.40	125.9 ± 3.5	155.2 ± 4.2

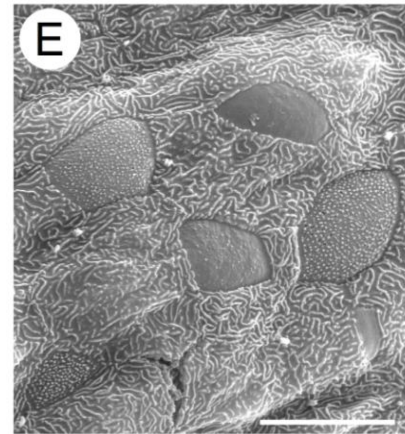
Footnote: All data are expressed as mean values +/- SEM (n= 20 for muscle moisture and serum Cl^-; n=11 for serum Na^+). No significant differences were detected between treatments.

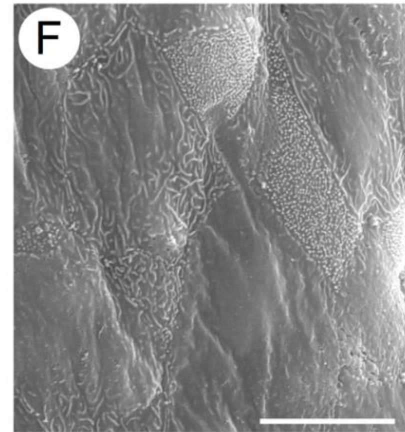












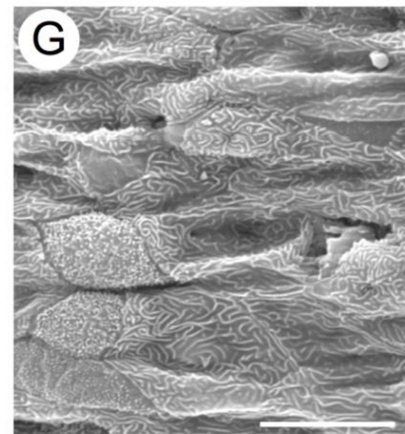


Figure 3: Effect of various dietary levels of fenugreek (5, 50, 500 µg fenugreek/g diet) on (A-C) rainbow trout gill mitochondria-rich cell (MRC) surface morphometrics. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control (ct). Values are expressed as mean +/- SE (n=5). Significant differences between treatment groups are indicated by different letters ($p<0.05$). Representative scanning electron micrographs for D) ct, E) 5, F) 50, G) 500µg/g fenugreek are shown. Scale bar represents 10µm.

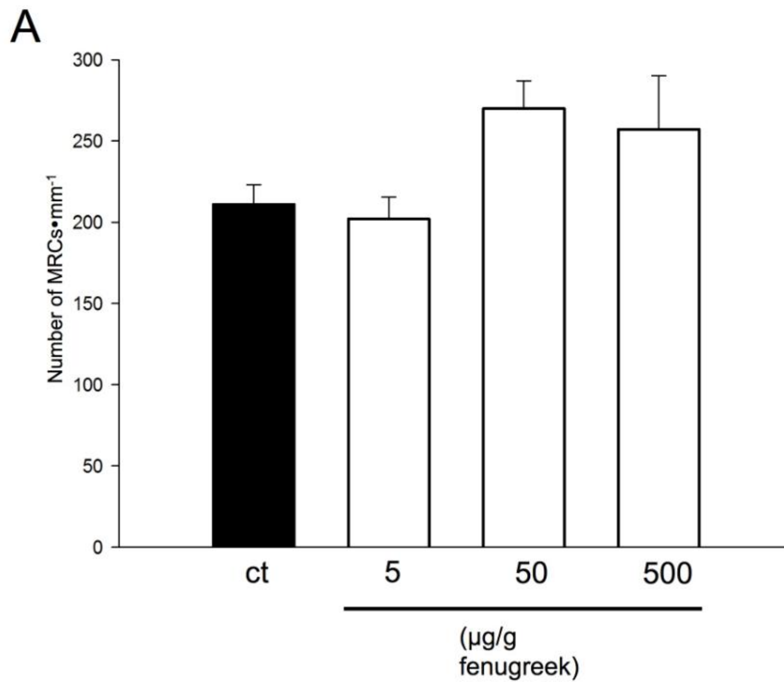
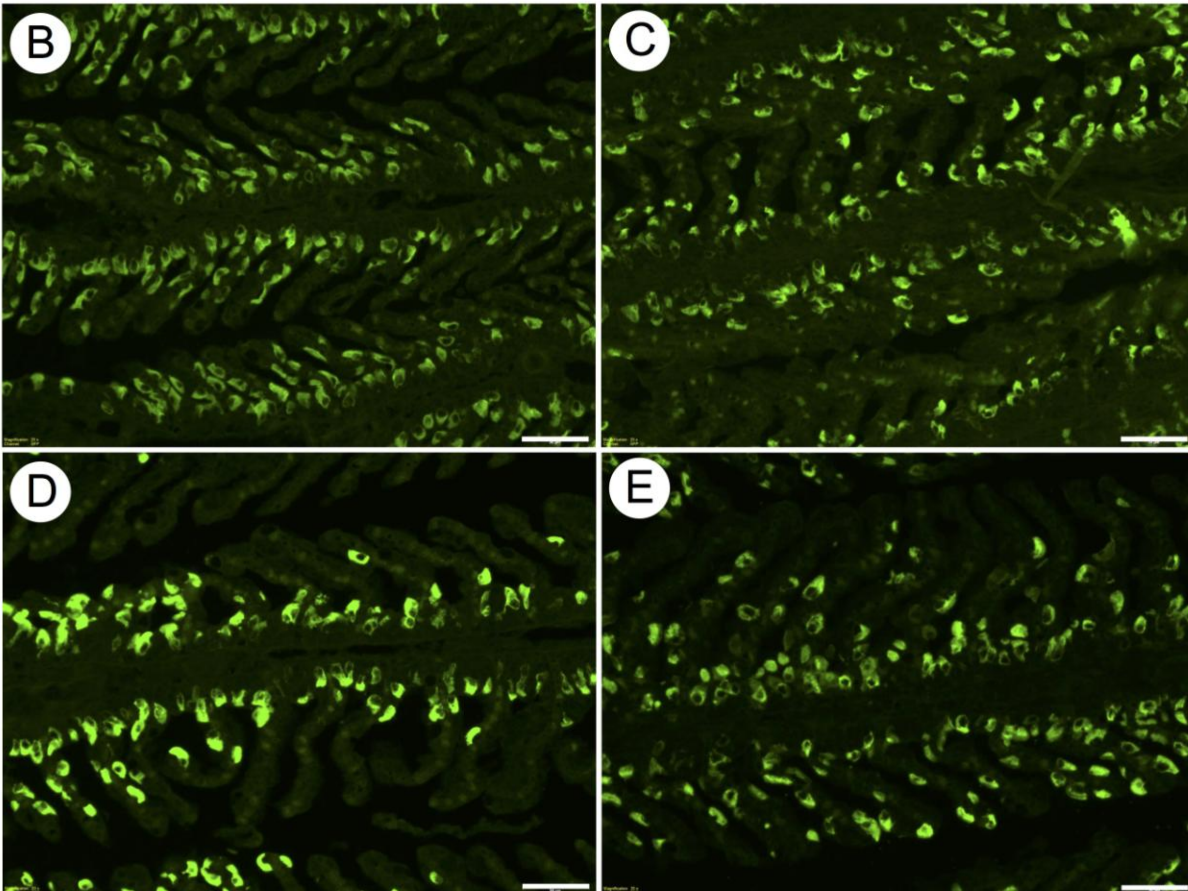


Figure 4: Effect of various dietary levels of fenugreek (5, 50, 500 µg fenugreek/g diet) on A) the average number of mitochondria-rich cells (MRCs) per mm of primary filament. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control (ct). Values are expressed as mean +/- SE (n=5). Representative images of Na^{+}-K^{+}-ATPase (NKA) immunreactivity are shown for B) ct, C) 5, D) 50, and E) 500 µg fenugreek/g diet. Scale bar represents 50µm.



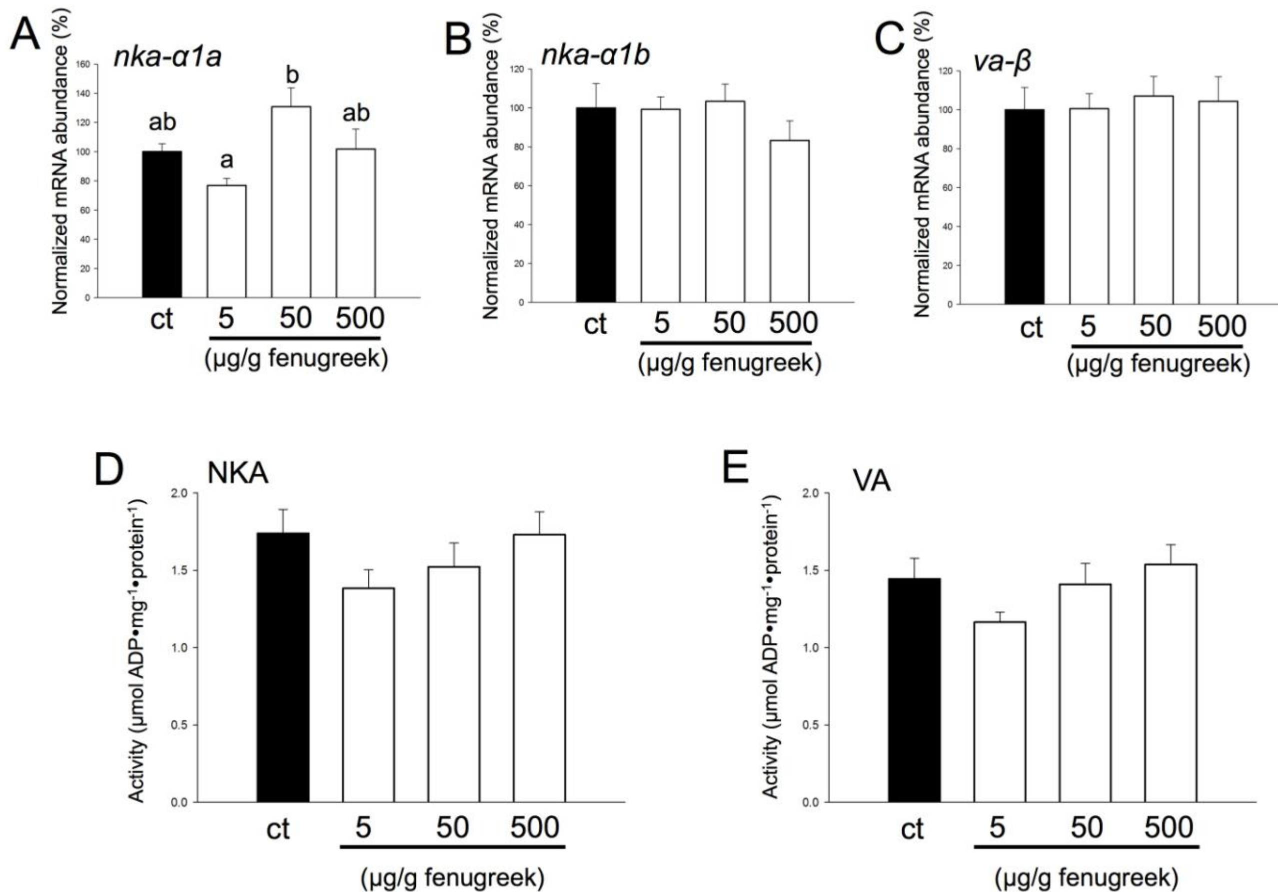


Figure 5: Effect of various dietary levels of fenugreek (5, 50, 500 µg fenugreek/g diet) on relative transcript abundance of Na^+-K^+-ATPase (NKA) subunits A) nka-α1a, B) nka-α1b, and the vacuolar-type H^+-ATPase subunit C) va-β. as well as on enzymatic activity of D) NKA and E) VA in the gill. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control (ct). Transcript abundance was normalized to elongation factor-1α as a reference gene. Transcript abundance was expressed relative to ct fish. Values are expressed as mean +/- SE (n=10). Significant differences between treatment groups are indicated by different letters ($p<0.05$).

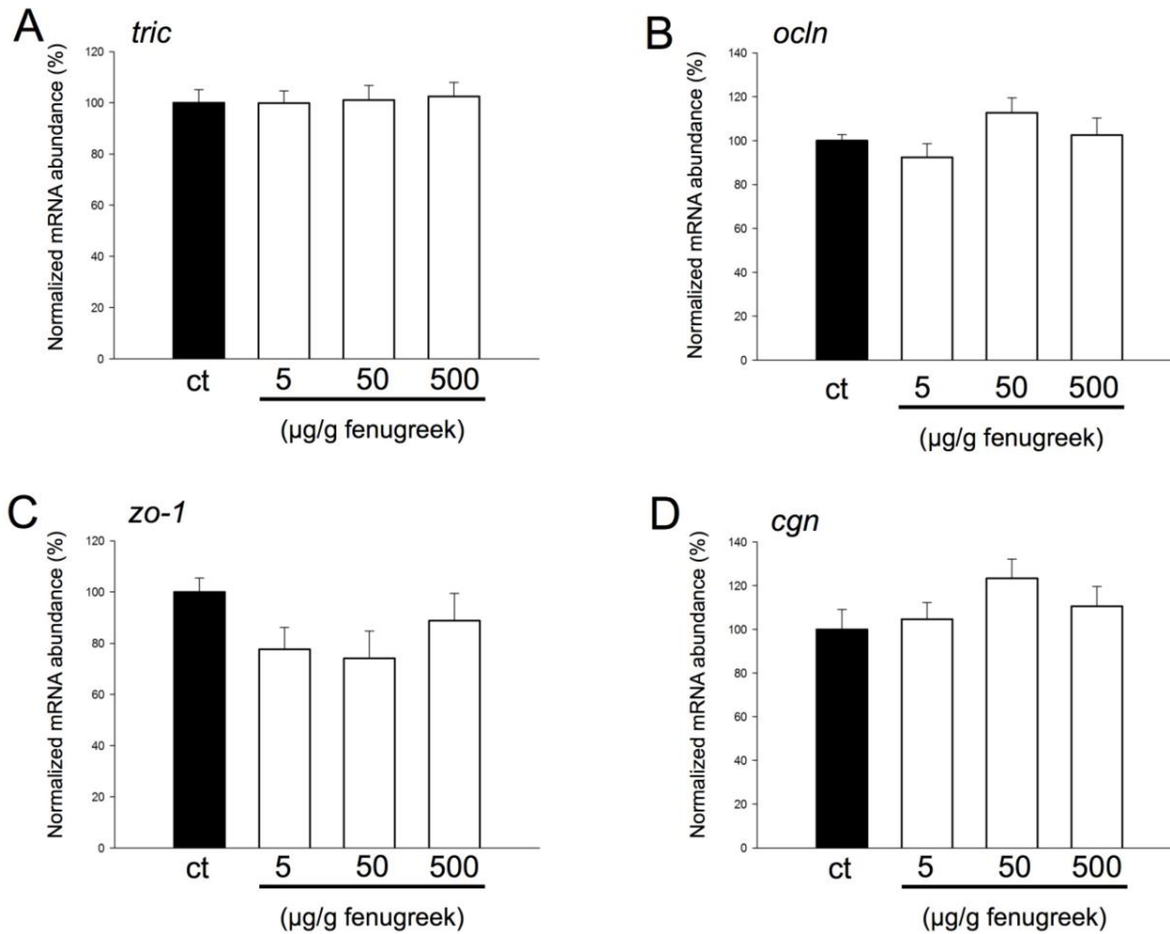


Figure 6: : Effect of various dietary levels of fenugreek (5, 50, 500 µg fenugreek/g diet) on relative transcript abundance of A) tricellulin (*tric*), B) occludin (*ocln*), C) zona-occludens-1 (*zo-1*), and D) cingulin (*cgn*) in the gill. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control (ct). Transcript abundance was normalized to elongation factor-1α as a reference gene. Transcript abundance was expressed relative to ct fish. Values are expressed as mean +/- SE (n=10).

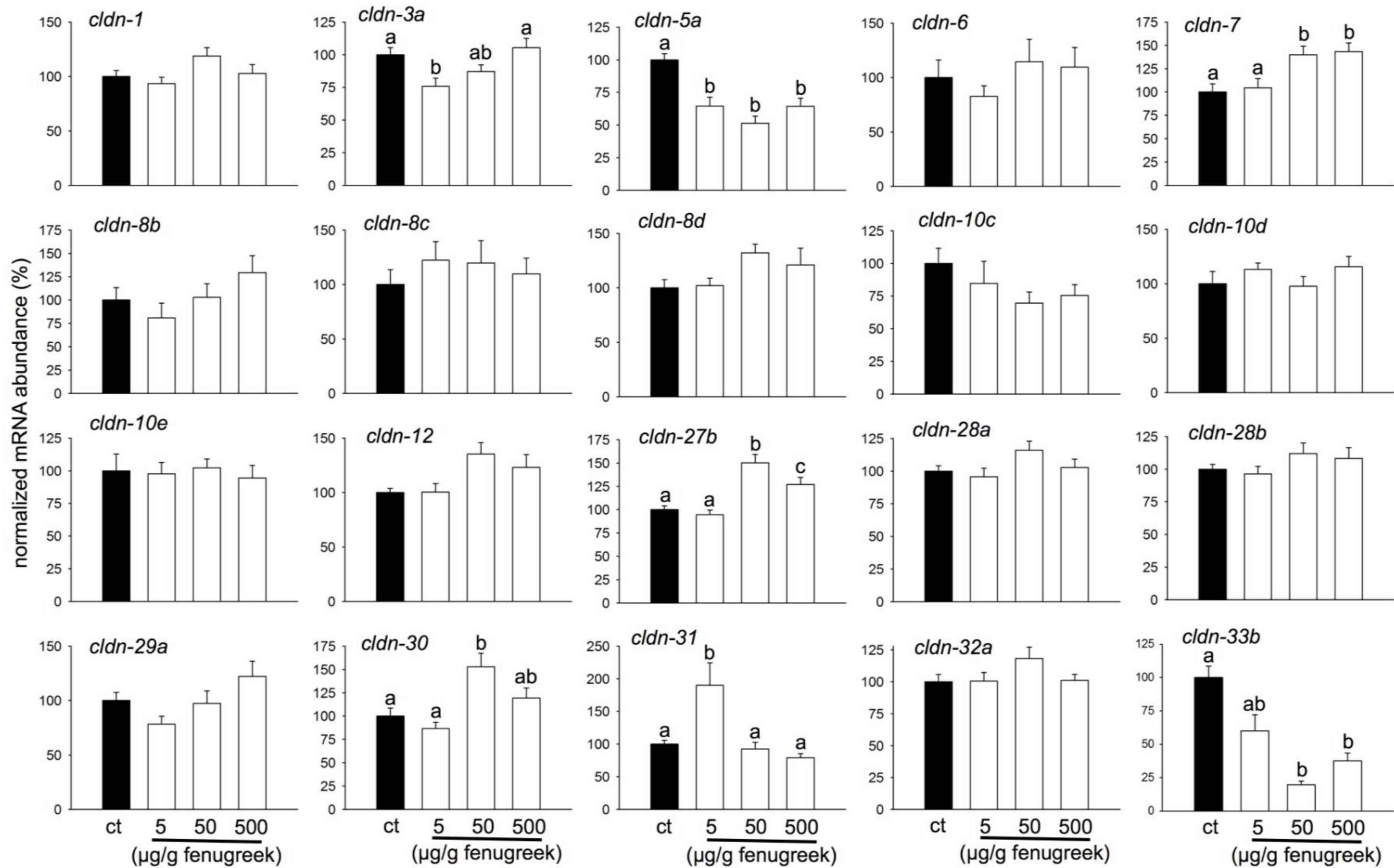


Figure 7: Effect of various dietary levels of fenugreek (5, 50, 500 µg fenugreek/g diet) on relative transcript abundance of the claudin (*cldn*) of tight junction (TJ) proteins in the gill. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control (ct). Transcript abundance was normalized to elongation factor-1α as a reference gene. Transcript abundance was expressed relative to ct fish. Values are expressed as mean +/- SE (n=10). No significant difference was seen in *cldn-23a* (not shown).

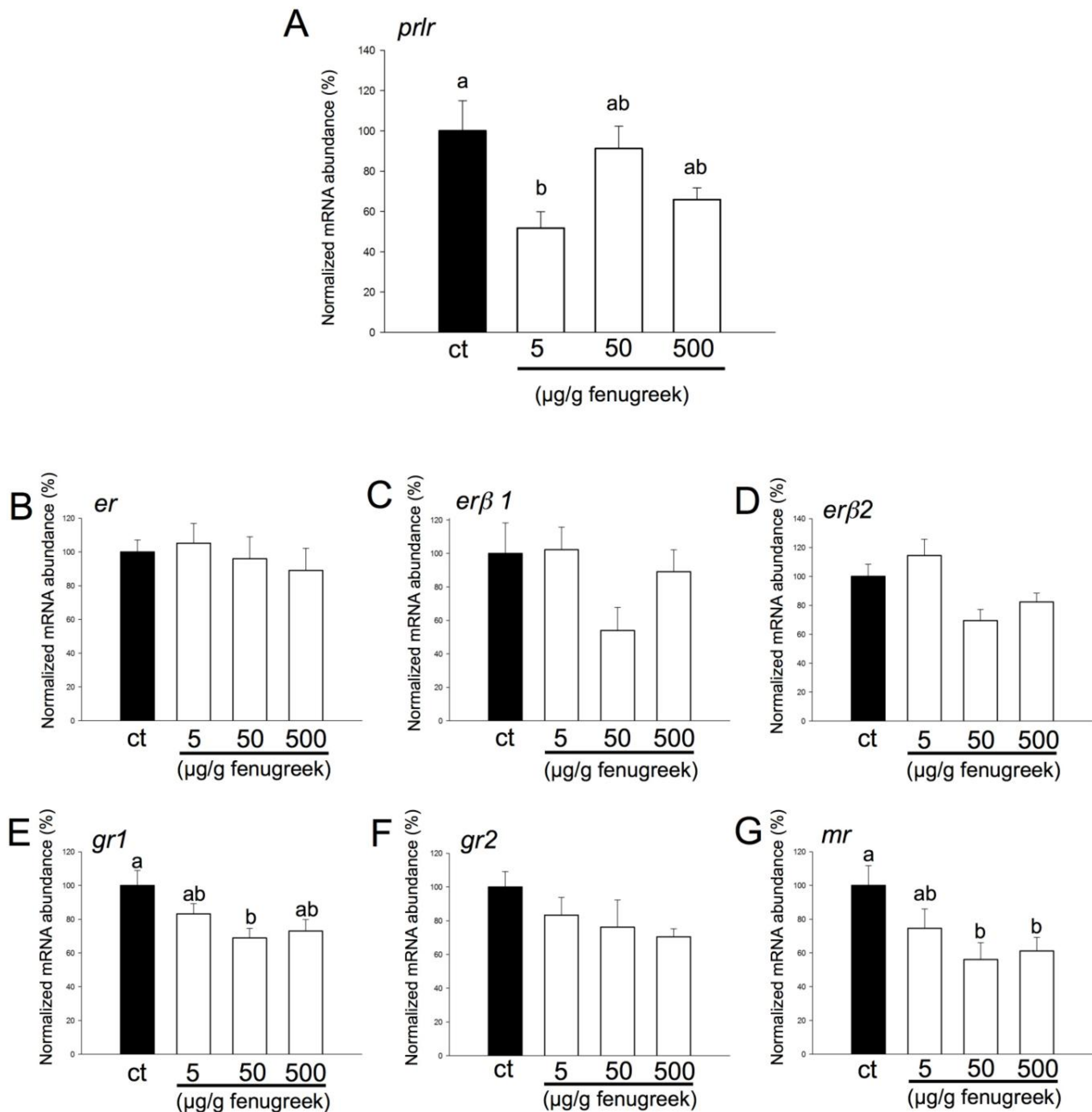


Figure 8: Effect of various dietary levels of fenugreek (5, 50, 500 µg fenugreek/g diet) on relative transcript abundance of the hormone receptors in the gill: A) prolactin receptor (*prlr*), B) estrogen receptor (*er*), C) *erβ1*, D) *erβ2*, E and F) glucocorticoid receptor-1 and 2 (*gr1, gr2*), and G) mineralocorticoid receptor (*mr*). A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control (ct). Transcript abundance was normalized to elongation factor-1α as a reference gene. Transcript abundance was expressed relative to ct fish. Values are expressed as mean +/- SE (n=10). Significant differences between treatment groups are indicated by different letters (p<0.05).

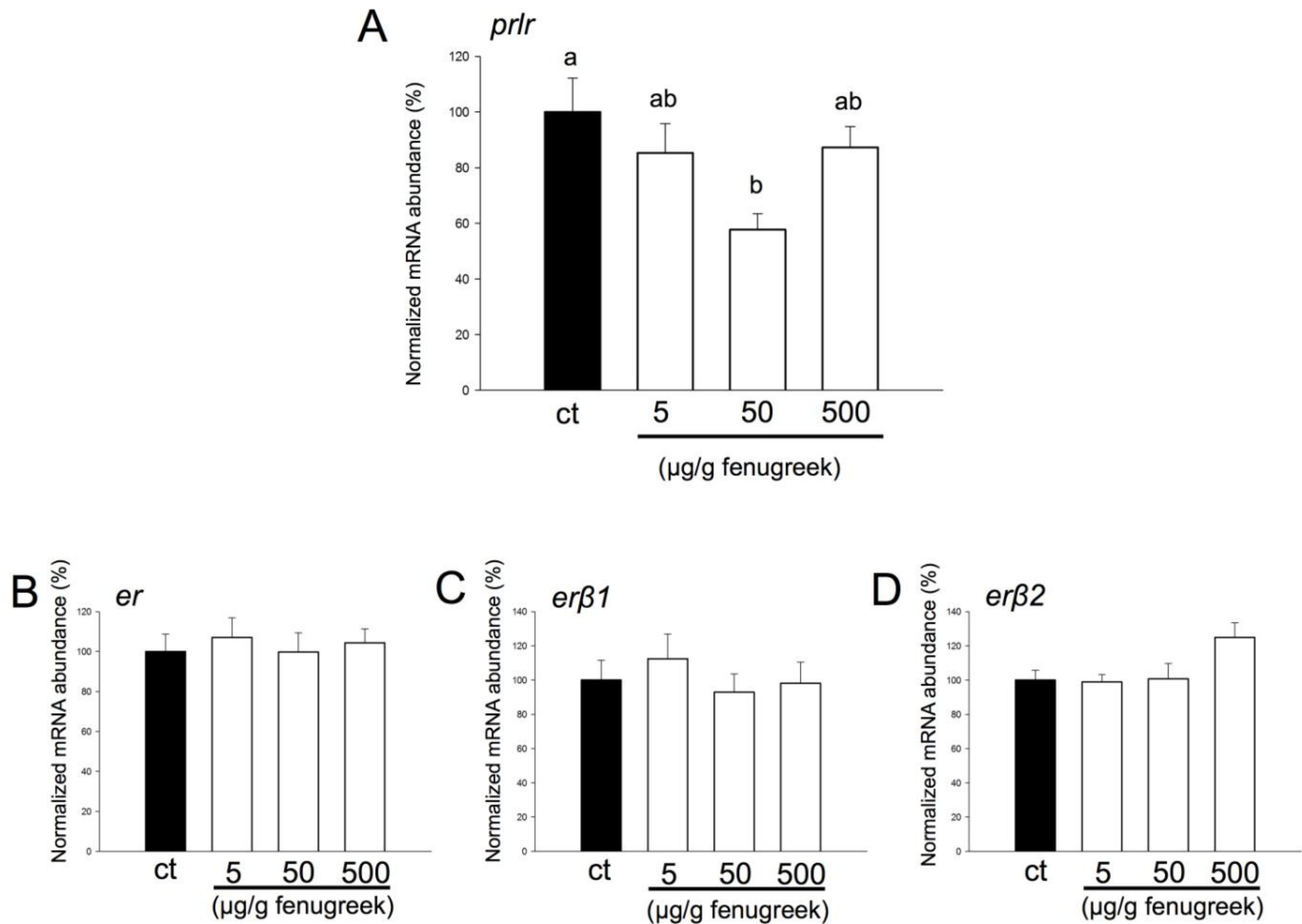


Figure 9: Effect of fenugreek on the relative abundance of hormone receptors in the hypothalamus of rainbow trout: A) prolactin receptor (*prlr*), B) estrogen receptor (*er*), C) *erβ1*, and D) *erβ2*. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control (ct). Transcript abundance was normalized to elongation factor-1α as a reference gene. Transcript abundance was expressed relative to ct fish. Values are expressed as mean +/- SE (n=10). Significant differences between treatment groups are indicated by different letters (p<0.05).

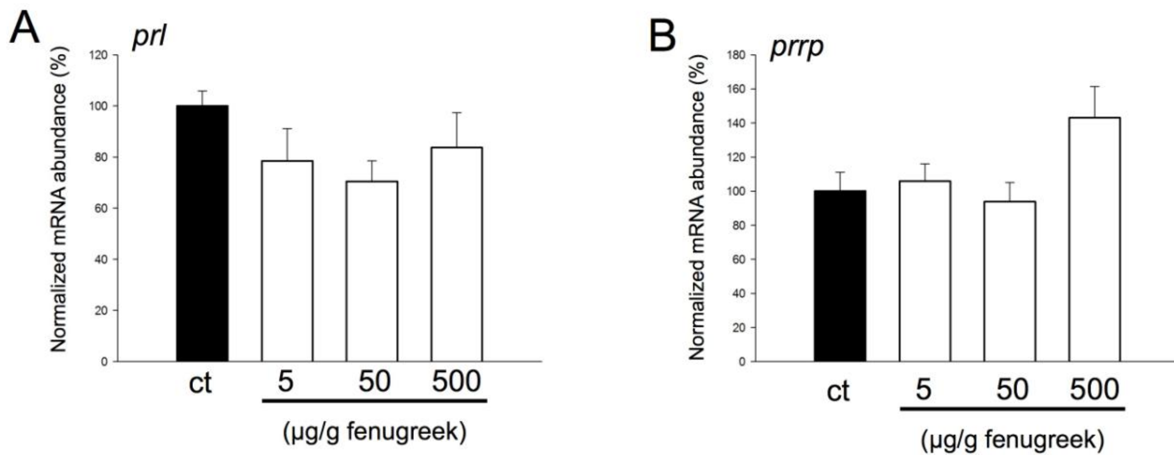


Figure 10: Effect of fenugreek on the relative abundance of A) *prl* abundance in the pituitary, and (B) *prrp* in the hypothalamus of rainbow trout. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control (ct). Transcript abundance was normalized to elongation factor-1α as a reference gene. Transcript abundance was expressed relative to ct fish. Values are expressed as mean +/- SE (A: n=6-8, B: n=10-12).

3.2 Effect of dietary fenugreek and IPW

3.2.1 Effect of dietary fenugreek and IPW on muscle moisture content, and serum [Cl^-] and [Na^+]

Following treatment with fenugreek and exposure to IPW, muscle moisture increased significantly in the IPW-exposed groups ($p=0.016$) (Table 4). Conversely, serum [Cl^-] decreased in IPW-exposed groups ($p<0.001$), and within IPW treated groups the fenugreek-treated group had significantly lower serum [Cl^-] than the control fed group ($p=0.013$) (Table 4). Serum [Na^+] also decreased significantly as a result of IPW exposure ($p<0.001$).

3.2.2 Effect of dietary fenugreek and IPW on gill MRC morphometrics

Exposure to IPW was found to decrease the number of MRCs and conversely increase the average size as measured by SEM (Fig. 11), however this difference was greater in the fenugreek-fed fish. Cell surface fractional area, being roughly the product of size and number of MRCs, did not change.

The number of MRCs per unit length of primary filament significantly increased with IPW exposure in the control diet fed fish as measured by IHC (Fig. 12). The number of MRCs increased as a result of fenugreek treatment in the FW group, however it decreased within the IPW group. The fenugreek-fed fish had similar MRC numbers across salinity treatments (Fig. 12).

3.2.3 Effect of dietary fenugreek and IPW on gill NKA & VA transcript abundance and activity

No significant changes were seen in ion transporter protein transcripts (Fig. 13 A-C).

Enzyme activity was not significantly affected by IPW, however fenugreek-fed fish gills had significantly lower enzyme activity for VA. No difference was seen in NKA, however there is a similar trend to VA was observed (Fig. 13 D,E).

3.2.4 Effect of fenugreek and IPW on TJ protein transcript abundance in the gill

Neither *tric* or *ocln* changed significantly. Of the scaffolding proteins in the gill, *zo-1* transcript levels decreased significantly within the FW group in the fenugreek-fed fish (Fig. 14), while *cgn* remained unchanged.

Of the 21 TJ protein transcripts examined, 10 Cldns exhibited significant changes with fenugreek and/or IPW exposure (Fig. 15). Changes in fenugreek-fed groups were observed in *cldn-1*, *cldn-5a*, and *cldn-8c.* Changes following IPW exposure were observed in *cldn-1, cldn-5a, cldn-10e*, *cldn-12*, *cldn-27b*, *cldn-30*, and *cldn-32a*. Interaction-dependent changes were observed in *cldn-28a*, and *cldn-33b*.

3.2.5 Effect of fenugreek and IPW on *prl, prrp,* and hormone receptor transcript abundance in the gill, pituitary, and hypothalamus tissue

Of the 7 hormone receptor transcripts quantified in the gill, 5 changed significantly with fenugreek and/or IPW exposure (Fig. 16). A significant decrease was seen in *er*β*2* as a result of IPW exposure. Fenugreek-fed fish exhibited lower transcript levels for *gr1*, *gr2*, and *mr*. Interaction-dependent changes were seen in *prlr*, and *er*β*2*. No changes were seen in *er* and *er*β*1*.

Of the endocrine receptor transcripts examined in the hypothalamus, 3 exhibited significant changes (Fig. 17). Fenugreek-fed fish differed in the

transcript abundance of *prlr* and *er*. Interaction-dependent effects were seen in *prlr* and *erβ2*.No changes were observed in *prl* or *prrp* transcript abundance measured in the pituitary and hypothalamus respectively (Fig. 18).

Table 4: Effect of fenugreek (50 μg fenugreek/g diet) on muscle moisture content, as well as serum [Cl^-] and serum [Na^+] in freshwater (FW) rainbow trout, and those abruptly exposed to ion-poor water (IPW) for 24 hours.

	μg/g diet	Muscle Moisture (%)	Serum [Cl^-] (mM)	Serum [Na^+] (mM)
FW	0	78.72 ± 0.22	135.6 ± 1.6	142.7 ± 5.2
	50	79.21 ± 0.17	135.9 ± 2.6	145.8 ± 7.2
IPW	0	79.12 ± 0.15 †	122.5 ± 2.2 †	116.7 ± 6.0 †
	50	79.53 ± 0.19 †	111.0 ± 4.0 †*	108.1 ± 7.0 †

Footnote: All data are expressed as mean values +/- SEM (n= 20 for muscle moisture and serum Cl^-; n=11 for serum Na^+). † denotes a significant difference due to IPW exposure, while * denotes significant effect of fenugreek (p<0.05).

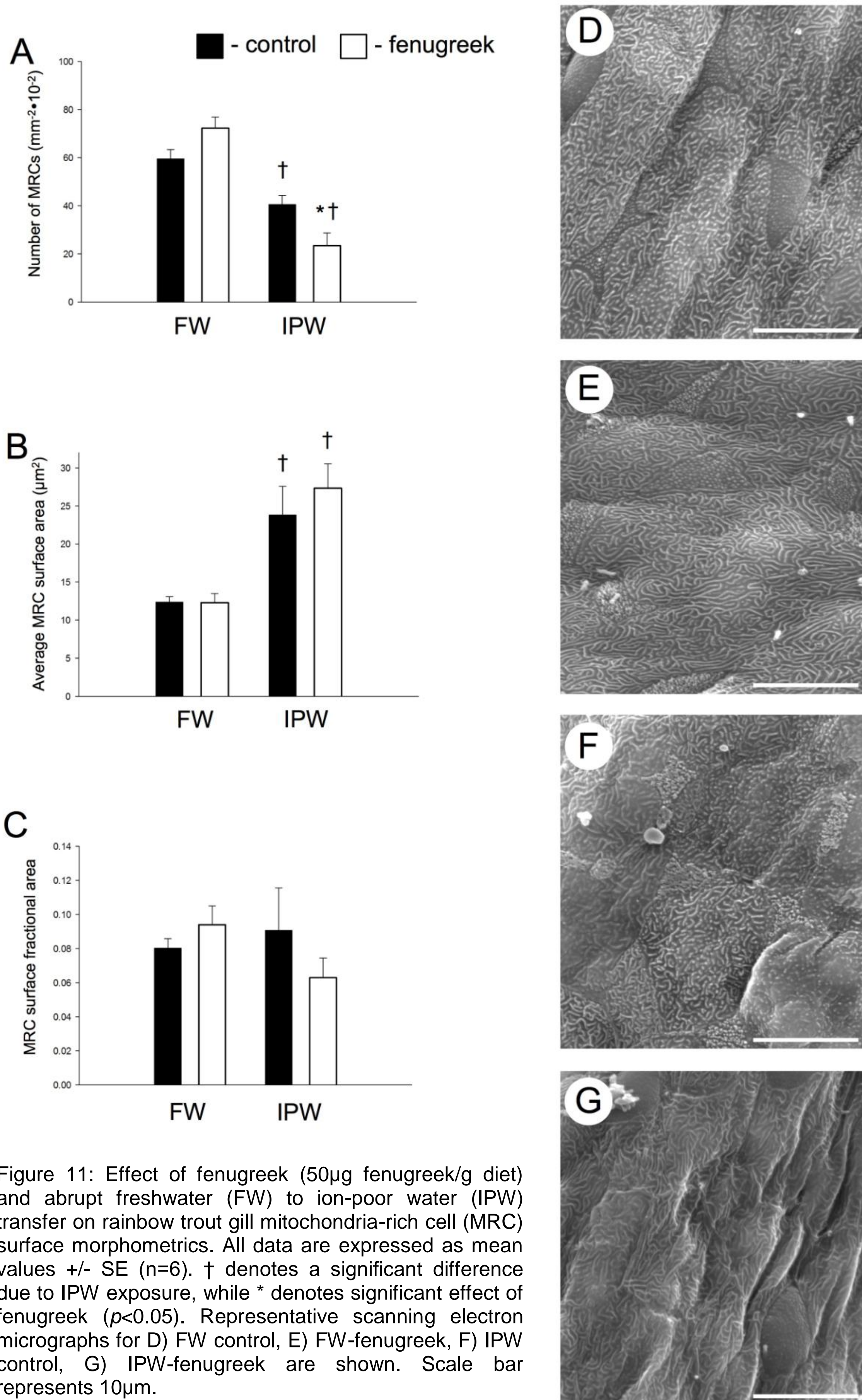


Figure 11: Effect of fenugreek (50μg fenugreek/g diet) and abrupt freshwater (FW) to ion-poor water (IPW) transfer on rainbow trout gill mitochondria-rich cell (MRC) surface morphometrics. All data are expressed as mean values +/- SE (n=6). † denotes a significant difference due to IPW exposure, while * denotes significant effect of fenugreek (*p*<0.05). Representative scanning electron micrographs for D) FW control, E) FW-fenugreek, F) IPW control, G) IPW-fenugreek are shown. Scale bar represents 10μm.

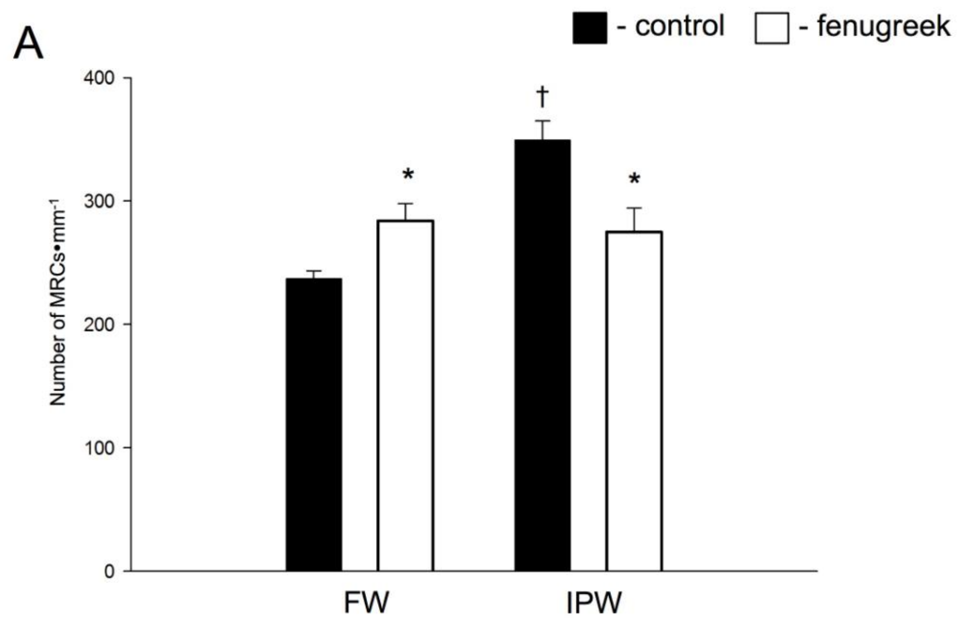
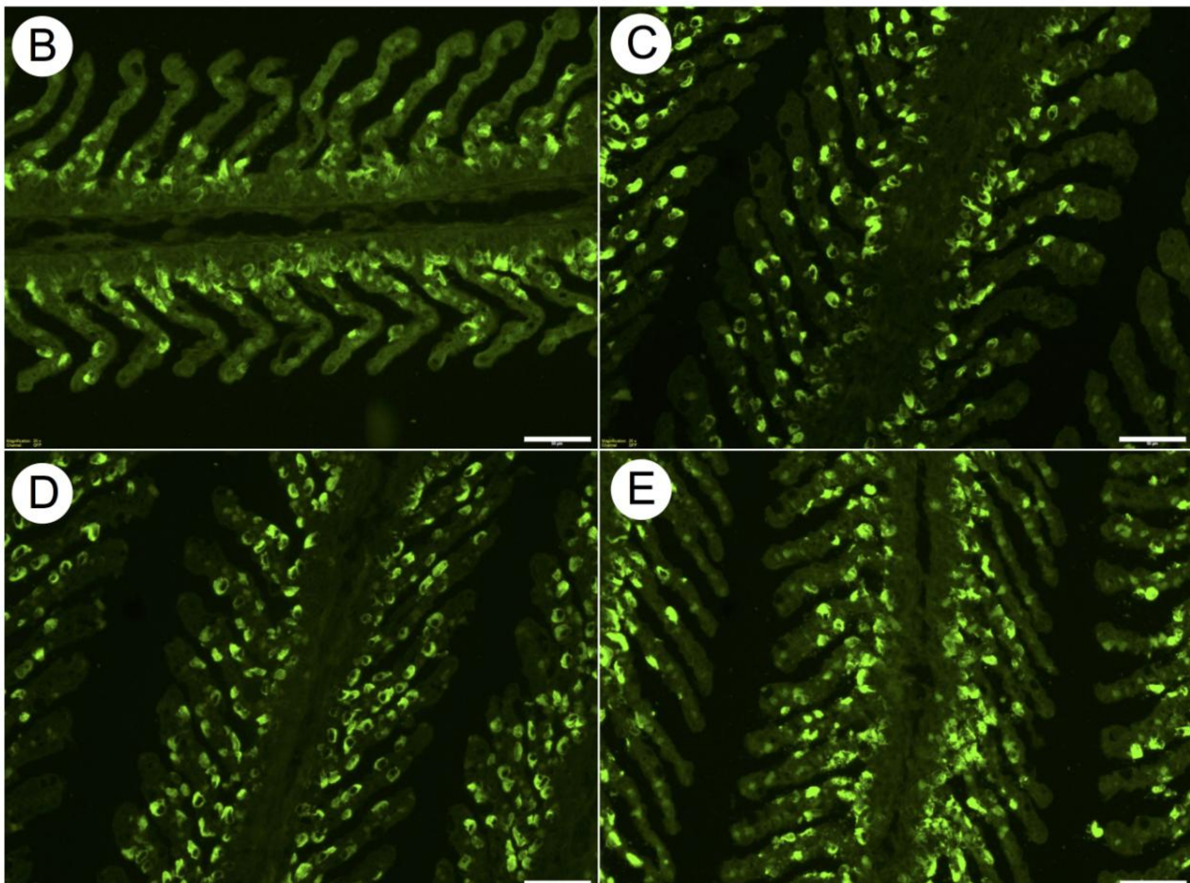


Figure 12: Effect of fenugreek (50µg fenugreek/g diet) and abrupt freshwater (FW) to ion-poor water (IPW) transfer A) the average number of mitochondria-rich cells (MRCs) per mm of primary filament. All data are expressed as mean values +/- SE (n=6). † denotes a significant difference due to IPW exposure, while * denotes significant effect of fenugreek (p<0.05). Representative images of Na^{+}-K^{+}-ATPase (NKA)-immunoreactivity for B) FW control, C) FW fenugreek, D) IPW control, E) IPW fenugreek are shown. Scale bar represents 50µm.



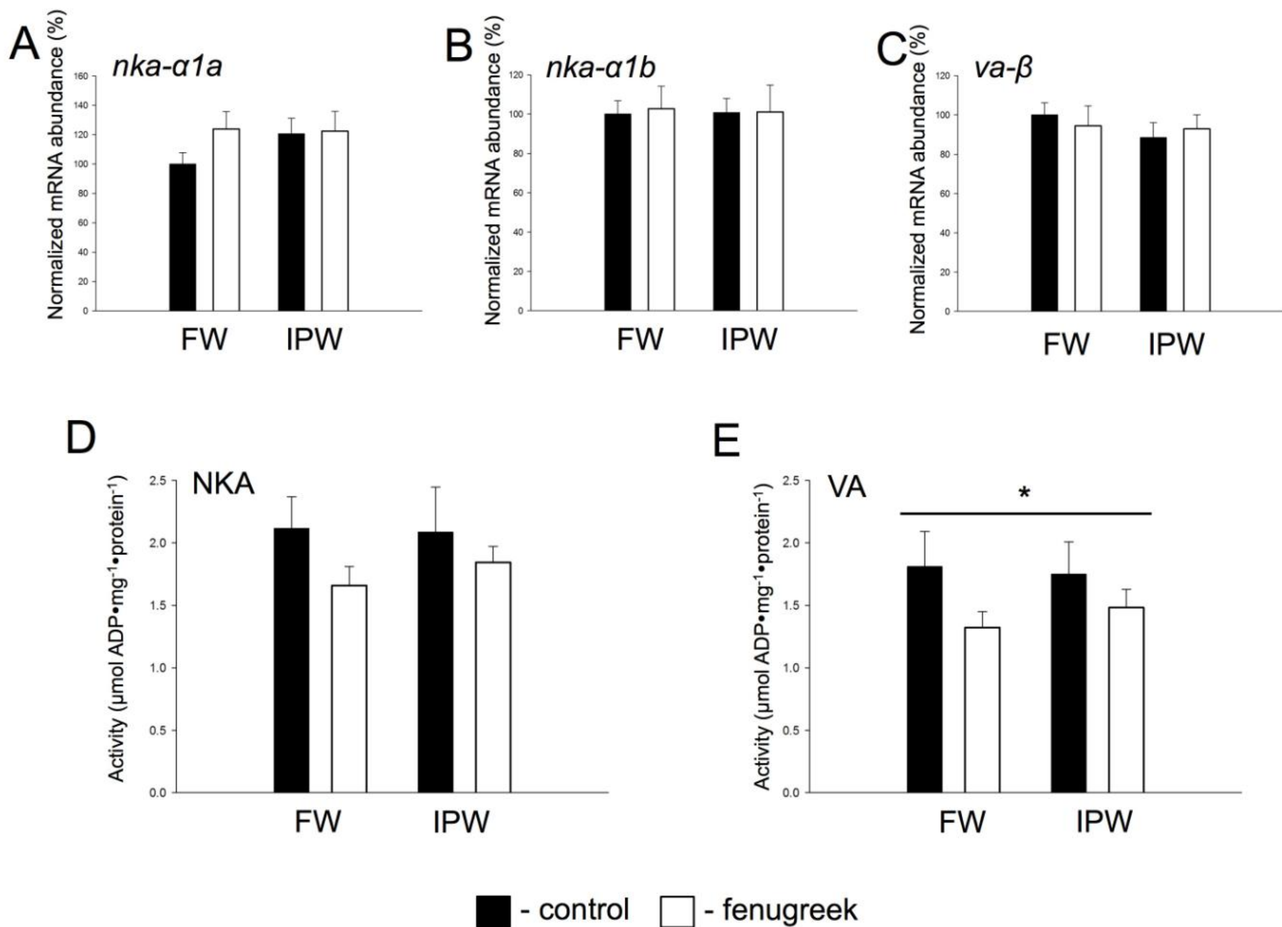


Figure 13: Effect of fenugreek (50µg fenugreek/g diet) and abrupt freshwater (FW) to ion-poor water (IPW) exposure on rainbow trout gill on the relative transcript abundance of Na^{+}-K^{+}-ATPase (NKA) subunits A) nka-α1a, B) nka-α1b, and the vacuolar-type H^{+}-ATPase subunit C) va-β as well as on enzymatic activity of D) NKA and E) VA in the gill. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control. Transcript abundance was normalized using β-actin as a reference gene. Transcript abundance is expressed relative to FW control fish assigned a value of 100%. Data are expressed as mean +/- SE (n=10-12). † denotes a significant difference due to IPW exposure, while * denotes significant effect of fenugreek (p<0.05).

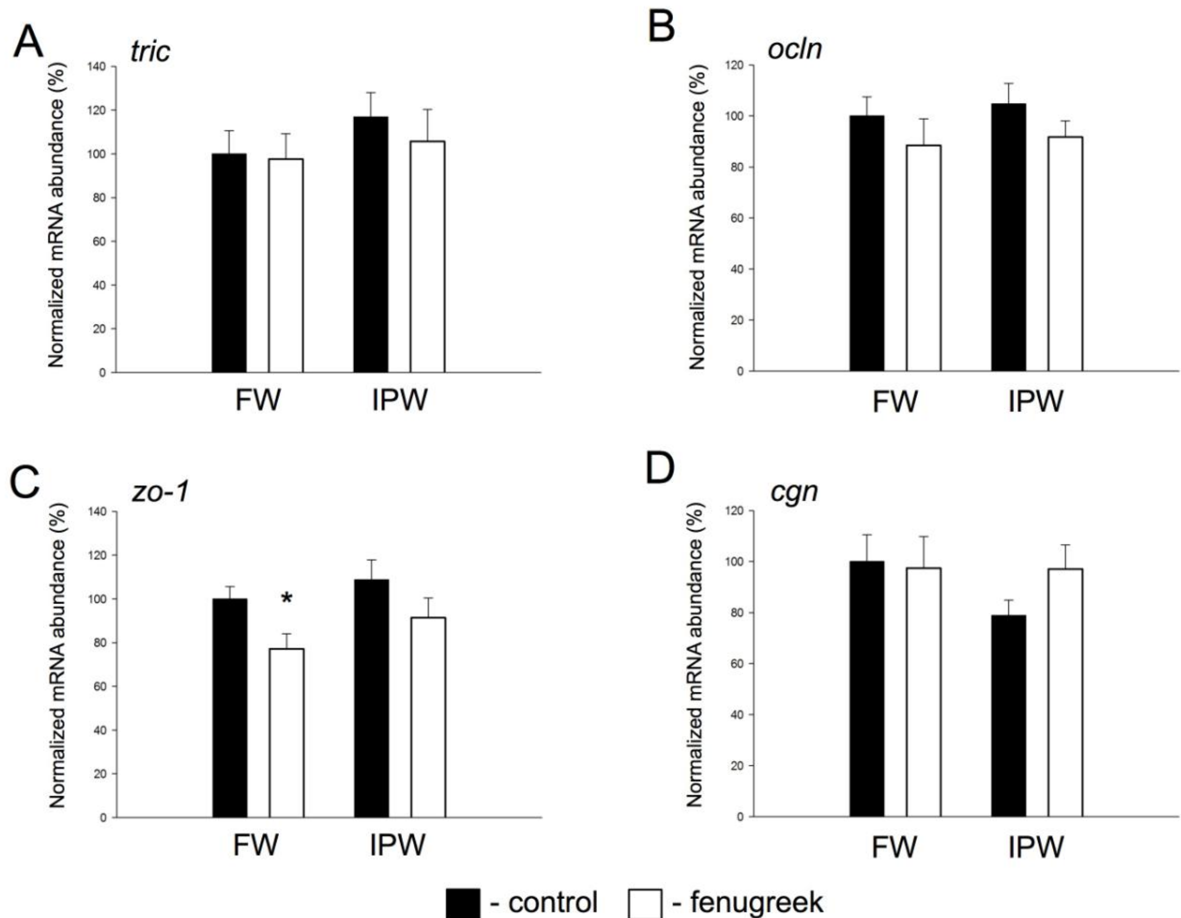


Figure 14: Effect of fenugreek (50μg fenugreek/g diet) and abrupt freshwater (FW) to ion-poor water (IPW) exposure on rainbow trout gill on the relative transcript abundance of A) tricellulin (*tric*), B) occludin (*ocln*), C) zona-occludens-1 (*zo-1*), and D) cingulin (*cgn*) in the gill. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control. Transcript abundance was normalized to elongation factor-1α as a reference gene. Transcript abundance was expressed relative to ct fish. Values are expressed as mean +/- SE (n=10-11). * denotes a significant difference due to diet ($p<0.05$).

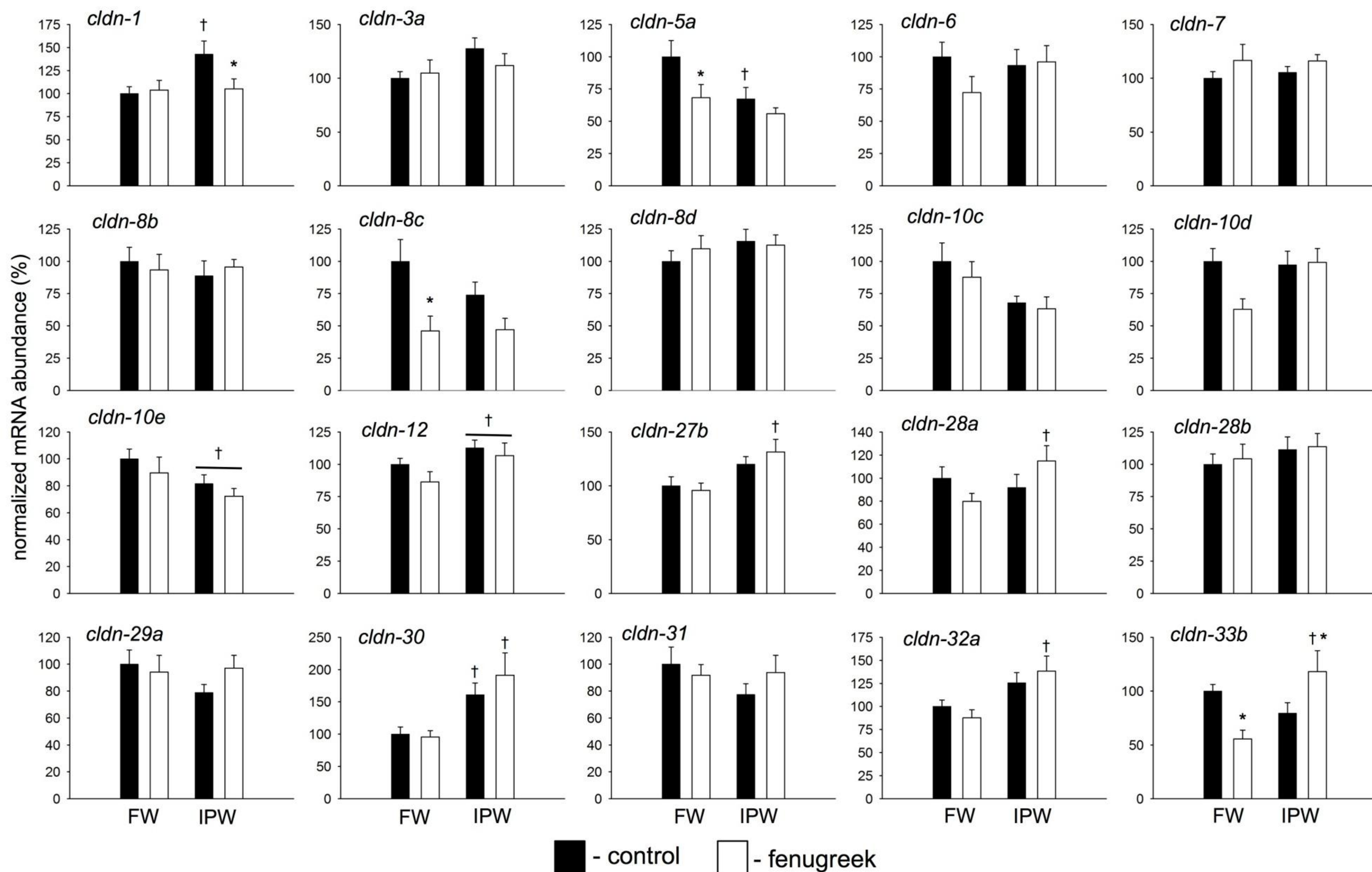


Figure 15: Effect of fenugreek (50µg fenugreek/g diet) and abrupt freshwater (FW) to ion-poor water (IPW) exposure on relative transcript abundance of the claudin (*cldn*) of tight junction (TJ) proteins in the gill. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control. Transcript abundance was normalized using β-actin as a reference gene. Transcript abundance is expressed relative to FW control fish assigned a value of 100%. Data are expressed as mean +/- SE (n=10-12). † denotes a significant difference due to IPW exposure, while * denotes significant effect of fenugreek ($p<0.05$). A bar denotes an overall difference. No significant difference was observed in *cldn-23a* (not shown).

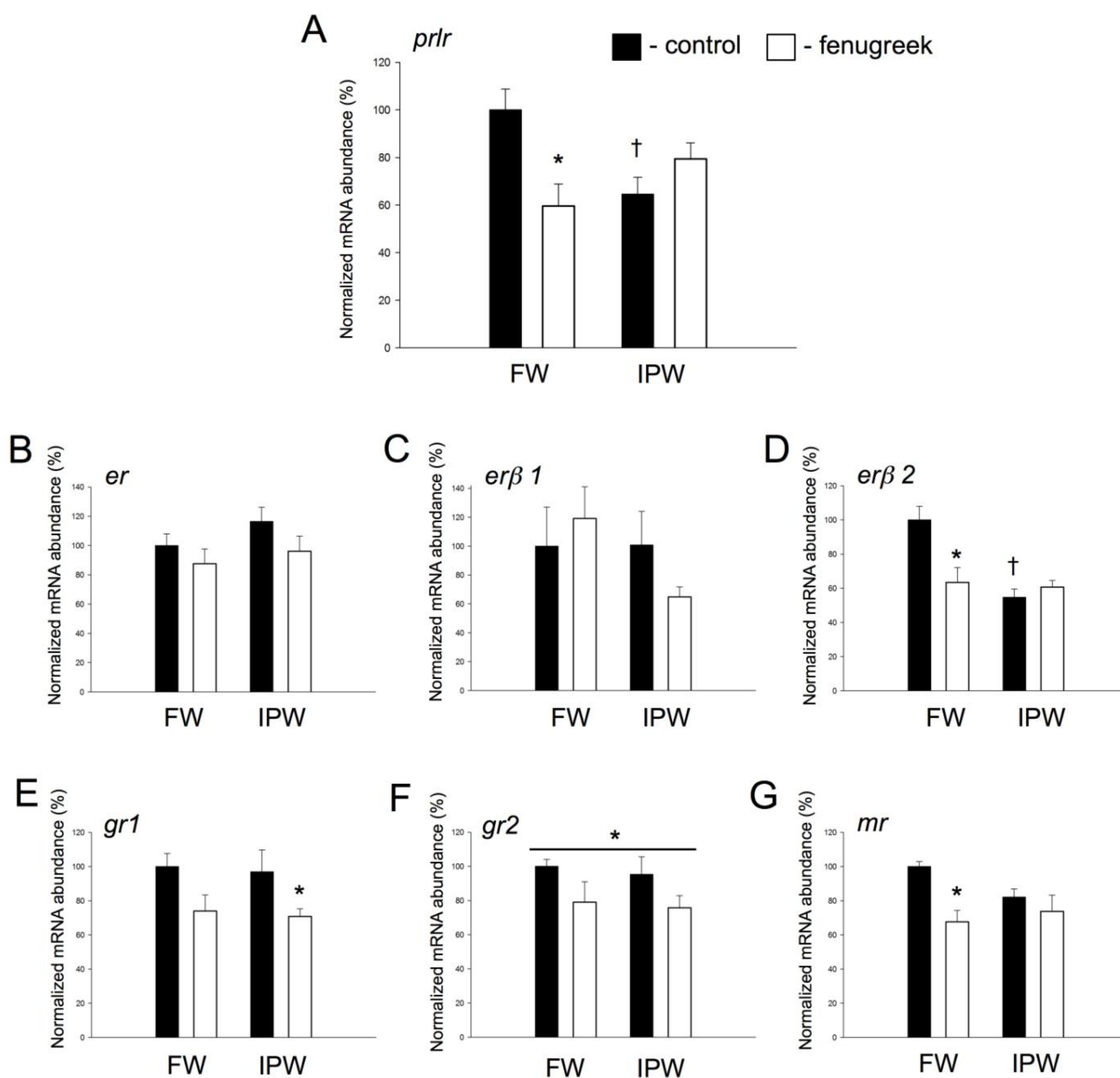


Figure 16: Effect of fenugreek (50μg fenugreek/g diet) and abrupt freshwater (FW) to ion-poor water (IPW) exposure on rainbow trout gill on the relative transcript abundance of the hormone receptors in the gill: A) prolactin receptor (*prlr*), B) estrogen receptor (*er*), C) *erβ1*, D) *erβ2*, E and F) glucocorticoid receptor-1 and 2 (*gr1, gr2*), and G) mineralocorticoid receptor (*mr*). A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control. Transcript abundance was normalized to elongation factor-1α as a reference gene. Transcript abundance was expressed relative to ct fish. Values are expressed as mean +/- SE (n=10-11). † denotes a significant difference due to IPW exposure, while * denotes significant effect of fenugreek ($p<0.05$). A bar denotes an overall difference.

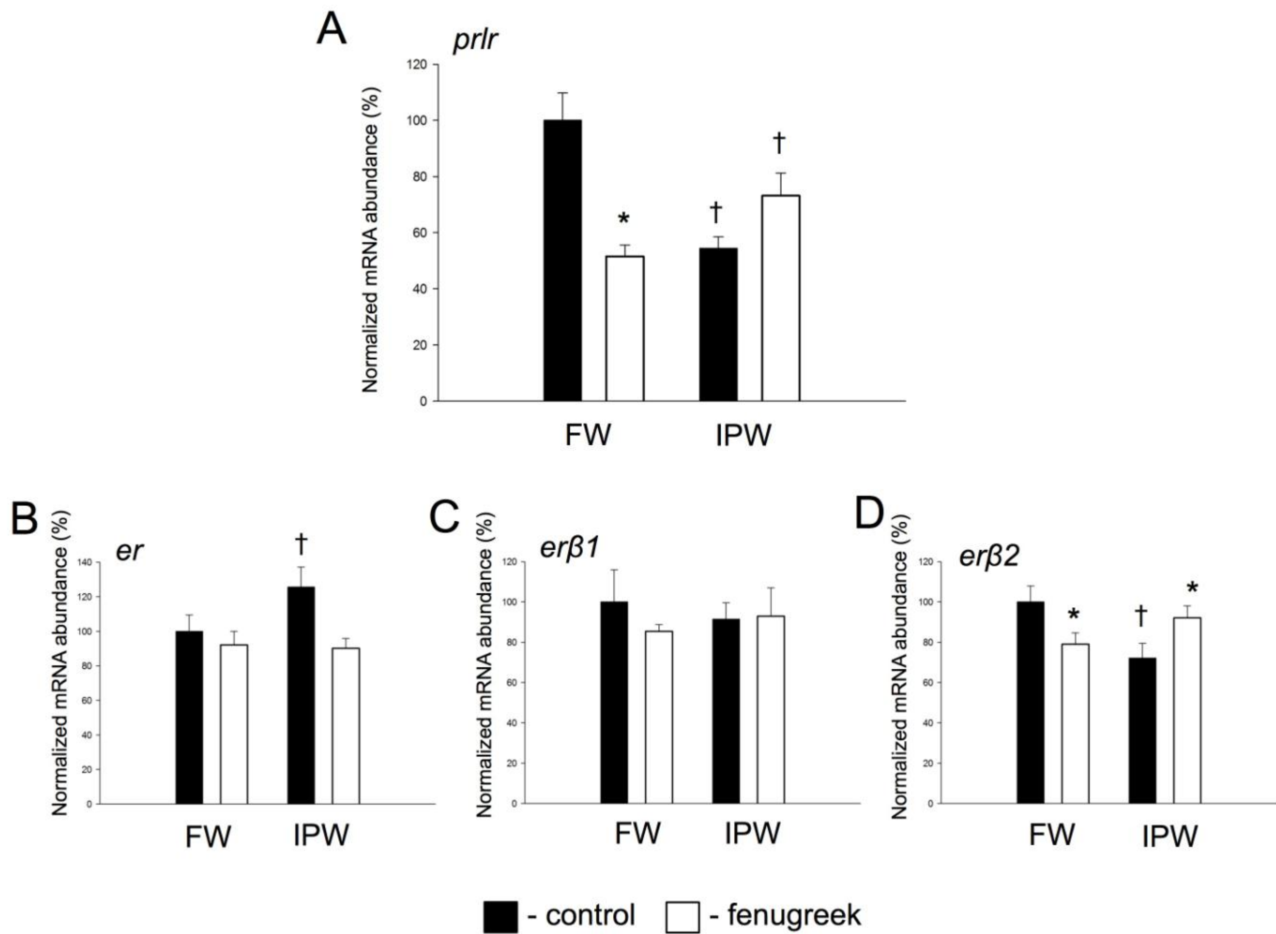


Figure 17: Effect of fenugreek (50μg fenugreek/g diet) and abrupt freshwater (FW) to ion-poor water (IPW) exposure on hormone receptors in the hypothalamus of rainbow trout: A) prolactin receptor (*prlr*), B) estrogen receptor (*er*), C) *erβ1*, and D) *erβ2*. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control. Transcript abundance was normalized to elongation factor-1α as a reference gene. Transcript abundance was expressed relative to ct fish. Values are expressed as mean +/- SE (n=10-11). † denotes a significant difference due to IPW exposure, while * denotes significant effect of fenugreek (p<0.05). A bar denotes an overall difference.

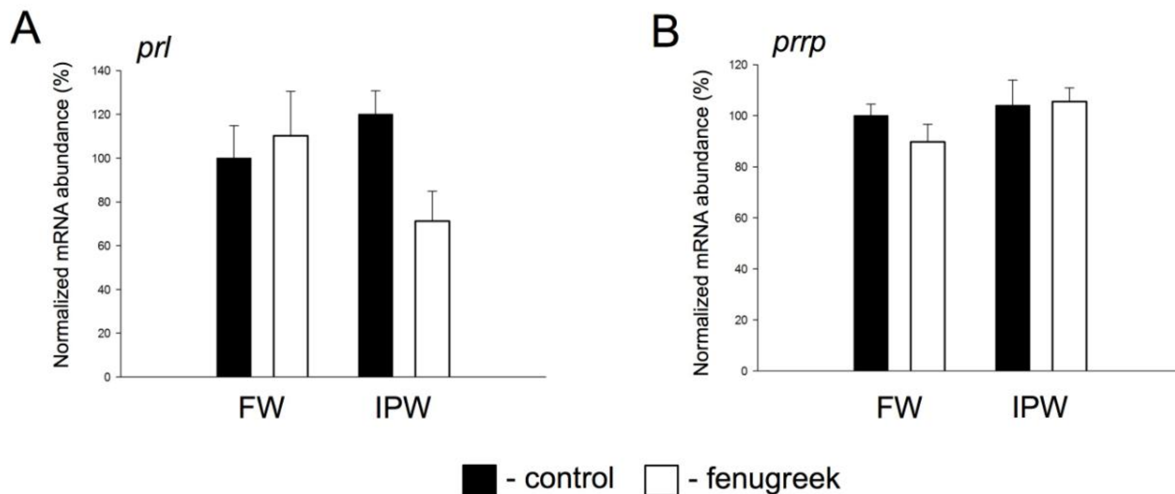


Figure 18: Effect of fenugreek (50μg fenugreek/g diet) and abrupt freshwater (FW) to ion-poor water (IPW) exposure on the relative transcript abundance of A) *prl* abundance in the pituitary, and (B) *prrp* in the hypothalamus of rainbow trout. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control. Transcript abundance was normalized to elongation factor-1α as a reference gene. Transcript abundance was expressed relative to ct fish. Values are expressed as mean +/- SE (n=10-11). † denotes a significant difference due to IPW exposure, while * denotes significant effect of fenugreek (p<0.05). A bar denotes an overall difference.

4.0 DISCUSSION

4.1 Overview

This study is the first to investigate the impact of a botanical galactogogue on salt and water balance in a teleost fish. While it was demonstrated that fenugreek has an impact on salt and water balance in the rainbow trout, the hypothesis that these changes would be brought about via Prl was not fully supported, given the absence of changes to *prl* and *prrp* transcripts. Despite maintaining ionoregulatory homeostasis as determined by analysis of serum [Na^+] and [Cl^-], and muscle moisture, dietary fenugreek did alter biochemical and molecular end points of salt and water balance, many of which are consistent with acclimation to FW.

4.2 Dose-dependent effect of fenugreek

Systemic end points of salt and water balance, such as serum sodium and chloride concentration, and muscle moisture content were unaltered in fish fed varying doses of dietary fenugreek (Table 3). This suggests that a normal physiological state was maintained. Consistent with these results, Prl treatment in FW acclimated chum salmon did not result in elevated plasma sodium levels (Hasegawa et al. 1986). In a previous study, Prl did not alter muscle moisture in SW-acclimated Atlantic salmon (Tipsmark et al. 2009). To the best of my knowledge, the effect of Prl on chloride levels on unhypophysectomized FW-acclimated fish has not been investigated. The absence of systemic changes with dietary fenugreek in normal (*i.e.* FW) conditions suggests that fenugreek is not deleterious in a manner that would result in excessive ion loss or reduced ion

uptake. However these results also suggest that in FW, fenugreek is not enhancing ionoregulation by promoting ion uptake or reducing ion loss. Nevertheless, the data to be discussed also indicates that changes in the morphology and molecular physiology of the gill epithelium are taking place. Therefore, these occur without significantly impacting salt and water balance in FW rainbow trout.

The number of MRCs exposed at the surface of the gill epithelium decreased in fish fed varying doses of dietary fenugreek (Fig. 3A). It is worth noting that, despite a lack of significance, there is a trend of increasing individual MRC surface area (Fig. 3B), which opposes the change seen in the number of exposed MRCs, thus the overall cell surface fractional area of MRCs is relatively consistent between groups (Fig. 3C). Laurent and Perry (1990) have shown a positive association between sodium and chloride fluxes and individual gill MRC surface area in rainbow trout treated with cortisol. However, it is worth noting that in this study, overall MRC surface fractional area increased significantly alongside individual MRC surface area, so it is uncertain whether individual MRC surface area would affect ion fluxes in the absence of MRC surface fractional area, as seen in the results presented here. In three species of salmonid acclimated to FW, the average size of MRCs increased following SW transfer, though no changes were seen in the number of MRCs (Hiroi and McCormick 2007). Thus hyperosmotic conditions have an opposite effect on the average size of MRCs compared to what was observed in fenugreek-fed fish. The effect of

Prl, in the absence of osmotic changes, on the number and size of exposed MRCs has not been investigated previously.

Despite detecting changes in the number of exposed MRCs at the surface of the gill epithelium using SEM, no significant changes in the density of MRCs were observed using IHC (Fig. 4). SEM images are localized to the proximal surface of the afferent edge of the primary filament, while IHC images focus on NKA-immunoreactive cells at and below the surface of the gill epithelium in the interlamellar region. Thus, differences seen between SEM and IHC may indicate an altered distribution of MRCs in the gill, however this is difficult to quantify using IHC and SEM.

The NKA subunit *nka-*α*1a*, which increased abundance in the 50 µg/g group, has also been shown to increase in abundance in FW relative to SW in salmonids (Richards et al. 2003, Bystriansky and Schulte 2011, Flores et al. 2012). While the exact purpose of the subunit switching is not clear, it is likely related to the need for ion uptake in FW, as opposed to ion secretion in SW. Thus, increased *nka-*α*1a* abundance in the gill may indicate that fenugreek can "prime" the gill for hypoosmotic conditions. Gill enzyme activity for NKA and VA did not change significantly (Fig. 5 D,E), however there is a trend of slightly lower activity levels in fenugreek-fed fish. Prolactin treatment alone did not change NKA activity in either hypophysectomized or sham fish in FW (Young et al. 1988).

In the present study, neither *ocln* nor *zo-1* showed observable changes with fenugreek treatment (Fig. 6). The tight junction protein Ocln becomes

elevated on the mRNA and protein level with cortisol treatment (Chasiotis et al. 2010). The scaffolding protein ZO-1 has exhibited decreased transcript abundance with cortisol treatment in the goldfish *Carassius auratus* (Chasiotis and Kelly 2011), however no changes were observed in rainbow trout primary cultured gill epithelium (Kelly and Chasiotis 2011). Since cortisol treatment is linked with a decrease in paracellular permeability, changes in transcripts of cortisol-linked proteins, such as Ocln and potentially ZO-1, may indicate a modification in paracellular permeability, however these changes are not necessarily induced by cortisol.

It has been shown previously that *cldn-3a*, *-7*, *-8d, -12, -28b, -30*, and *-31* transcript levels become elevated with cortisol treatment, and thus, these Cldns are also associated with decreased paracellular permeability (Kelly and Chasiotis 2011). Of these Cldns, *cldn-3a*, *-7*, *-30*, and *-31* changed in the gill (Fig. 7). In *cldn*-7, *-30*, and *-31*, the transcript levels were elevated in fenugreek-treated groups, which, if reflected on the protein level, may indicate reduced paracellular permeability. Two of these are the most abundant Cldn transcripts examined in the gill, *cldn-7* and *cldn-30*. Cldn-30 transcript abundance has been shown to decrease during SW acclimation in *Salmo salar* (Tipsmark et al 2008), and has been associated with decreased permeability to monovalent cations such as sodium (Engelund et al. 2012). These data suggest that a FW acclimation response may be occurring in *cldn-30*. However no changes were seen in *cldn-30* abundance with Prl injection (Tipsmark et al. 2009). While many of these

Cldns have been linked with decreased paracellular permeability, it is unknown whether these changes have resulted in decreased permeability in this case.

The transcript of *cldn-33b* is present in gill tissue, but absent from PVC-only primary gill cell culture preparations (Kolosov et al. 2014), so it likely to be associated with another cell type in the gill. Thus, the changes observed in the transcript levels of *cldn-33b* (Fig. 7) might reflect alterations in the abundance or surface area of a particular cell type. Kolosov et al. (2014) speculated that an association between *cldn-33b* and MRCs would be unlikely based on the discrepancy between the expected and observed transcript abundance (Fletcher et al. 2000). Therefore it is likely to be associated with another gill cell type, such as mucus cells, neuroepithelial cells, or vasculature. Regardless, this potential specificity is intriguing, as it implies a specialized role. No changes were observed in *cldn-10* transcripts as a result of dietary fenugreek. Cldn-10d has been shown to associate with MRCs in pufferfish (Bui and Kelly 2014), and *cldn-10e* is absent in PVC-only cell cultures in pufferfish, and decreases over time in rainbow trout PVC cultures (Bui and Kelly 2014, Kolosov et al. 2014). The abundance of both Cldn transcripts increased in SW relative to FW. Transcript levels of *cldn-28a* remained unchanged with fenugreek treatment, although it has been previously shown to increase with Prl treatment in SW-acclimated Atlantic salmon (Tipsmark et al. 2009). Similarly, transcript levels of *cldn-28b*, a Cldn shown to decrease in transcript abundance in response to both hypoosmotic and hyperosmotic stress (Tipsmark et al. 2008, Tipsmark et al 2009, Sandbichler et al. 2011), were also unchanged.

Gill *prlr* transcript abundance in sockeye salmon *Oncorhynchus nerka* was seen to correlate strongly with *nka-α1a* abundance, and both transcripts increased throughout FW migration (Flores et al. 2012). However this correlation was not seen in this study, as *prlr* decreased significantly, however only in the 5 µg/g group, whereas *nka-α1a* only increased in the 50 µg/g group (Figures 5 and 8). The decoupling of *nka-α1a* and *prlr* is an indication that if fenugreek does induce a FW acclimation response in rainbow trout, that it may only be a partial response.

In this study, no changes were observed in rainbow trout gill estrogen receptors in fenugreek-fed fish (Fig. 8). Of the five documented ERs (ER, ERα2, ERβ, ERβ1, and ERβ2) in rainbow trout, only transcripts of the three presented here (ER, ERβ1, ERβ2) were found in the gill in quantifiable levels (Nagler et al. 2007), and thus the more likely candidates for involvement in osmoregulation. ERα and ERβ are both nuclear estrogen receptor subtypes in rainbow trout, acting as transcription factors (Nagler et al. 2007). Estrogen receptors in the membrane are regulated by estrogens; either via gene expression, trafficking or insertion/internalization into the membrane (Dominguez and Micevych 2010, Gorosito et al. 2008). The absence of differences in estrogen receptor transcripts is not an indication that its levels in the membrane are not changing, though there is a possibility of other types of regulation acting on estrogen receptors.

The corticosteroid receptors *gr1* and *mr* both decreased in fenugreek-fed fish (Fig. 8). Blocking of the mineralocorticoid receptor (MR) by spironolactone inhibits MRC proliferation in IPW exposed rainbow trout (Sloman et al. 2001).

Similarly, GR levels are positively correlated with increased NKA activity in response to cortisol exposure (Shrimpton and McCormick 1999). Thus, if reflected on the protein level, the decreased transcript abundance of corticosteroid receptors has the potential to mute alterations to NKA activity in MRCs and paracellular permeability in response to corticosteroids. Glucocorticoids have been shown to depress transcript levels of *gr* (Rosewicz et al. 1988), so it is possible that the depression seen in corticosteroid receptors in fenugreek-fed fish is linked to some ligand binding the receptors, either through promoting the release of cortisol, or a component of fenugreek extract binding the receptors.

Consistent with the gill, *prlr* levels in the hypothalamus decreased significantly (Fig. 9). This further supports the decoupling of the correlation between *prlr* and *nka-*α*1a*. Similarly consistent with the gill, estrogen receptor transcript levels did not change, however it is difficult to draw conclusions about this due to the non-transcriptional forms of regulation of estrogen receptor levels.

Prl and PrRP transcript abundance in the pituitary and hypothalamus respectively, remained unchanged with fenugreek treatment (Fig. 10). PrRP, as the name suggests, is known to increase circulating levels and transcript abundance of Prl in rainbow trout (Sakamoto et al. 2003). Following FW transfer, Prl levels are shown to increase and remain elevated for at least a 3-week period (Prunet et al. 1985). Thus, if fenugreek had caused a change in Prl, it is likely that it would be observed over the length of this study, although transcript levels

do not always correspond with protein levels. Therefore it seems unlikely that fenugreek treatment has an effect on Prl, either directly or upstream.

4.3 Effect of dietary fenugreek and IPW

Organisms living in hypoosmotic environments face a constant efflux of ions and an influx of water according to their respective gradients. In this respect, IPW exposure is expected, and has been previously shown to intensify these challenges relative to normal FW (Chasiotis et al. 2008, Chasiotis et al. 2012a). This expectation was confirmed in the increased moisture content of muscle tissue and decreased serum [Cl^-] and [Na^+] in IPW-exposed fish relative to FW-acclimated fish (Table 4). No changes were seen as a result of dietary intake of fenugreek with regard to muscle moisture and serum [Na^+], however in the case of serum [Cl^-], fenugreek exacerbated the effect of IPW. Because of the closely maintained balance of salt and water, the changes seen in these physiological end points in IPW-exposed groups are assumed to indicate suboptimal conditions. Thus the changes that fenugreek brings about in the biochemistry and molecular physiology of the rainbow trout do not enhance its ability to cope with an abrupt exposure to IPW as initially hypothesized.

As determined by SEM, IPW treatment decreased the number of exposed MRCs, while increasing the average area of apical exposure of MRCs in the gill epithelium (Fig. 11). These changes qualitatively mimic the effect of fenugreek in the dose-response experiment; however in this experiment, fenugreek-fed fish in FW did not exhibit any significant morphometric changes in the gill relative to control diet-fed fish. In IPW, fish fed varying doses of dietary fenugreek had

significantly fewer exposed MRCs than their control diet counterparts. In effect, dietary fenugreek intensified the IPW response. While perturbations in physiological end points indicate a suboptimal state, in the case of morphological changes the exaggeration of the IPW response with fenugreek treatment may point to an enhanced response. This is because morphological changes are likely reflective of a coping response. In rainbow trout, both total and individual surface area of MRCs have been shown to increase gradually and steadily following IPW transfer (Perry and Laurent 1989, Greco et al. 1996). Similarly, both MRC number and size increased with IPW exposure in goldfish (Chasiotis et al. 2012b). The data presented here are only partially in agreement with previous experiments, in that the average area of exposure of individual MRCS also increased, while the number of exposed MRCs was shown to decrease. However it is worth noting that the timeframe of IPW acclimation of the listed studies ranges from 4 days to 30 days. The significance of increased size and decreased number is unknown, as the fractional surface area in contact with the external media is similar between treatments. These changes would result in a reduced apical junction distance (*i.e.* "circumference" of the cell surface) to surface area ratio, so any MRC-specific Cldns, discussed later, would become less abundant.

Consistent with the dose-response experiment, an increase in the number of MRCs was observed using IHC as a result of fenugreek in the FW-exposed fish (Fig. 12). The largest increase in the number of MRCs was seen in control diet-fed fish exposed to IPW. The number of MRCs is similar in fenugreek-fed

fish regardless of salinity treatments, which indicates that the effect of fenugreek may be mostly overriding the effect of IPW. IPW-acclimated rainbow trout exhibited an overall increase in the number of MRCs exposed, as well as a major redistribution of MRCs to the lamellae relative to fish kept in FW (Laurent and Hebibi 1989). This relationship is consistent with the decrease in the number of lamellar MRCs in SW-acclimated Atlantic salmon (Hiroi and McCormick 2007). In IPW, the lamellae increase in length, measured from primary filament to the tip of the lamella (Laurent and Hebibi 1989). Thus it is likely that lamellar MRCs are associated with ion uptake in FW, however no distribution analyses were conducted in this study.

While no significant changes were seen in *nka-*α*1a*, the FW-subunit of NKA, there was a trend of an increase in transcript abundance in all treatments relative to FW-control (Fig. 13). The absence of changes seen in NKA activity with regard to the effect of IPW is consistent with prior research in the sockeye salmon in acute and chronic transfers (Flores and Shrimpton 2012). Similar results were seen in VA activity with regard to the effect of IPW. The decrease in VA activity in fenugreek-fed fish may point to a reduced need for active ion uptake. It is worth noting that NKA activity follows a similar qualitative trend as VA, however the changes were not significant. Reduced enzyme activity could indicate that fenugreek-fed fish have a reduced need for ion uptake. However, this notion is contradicted by the consistent levels of serum [Na^+] and muscle moisture observed between control and fenugreek-fed fish in IPW treatments Reduced active ion uptake could explain the lower serum [Cl^-] in fenugreek-fed

fish in IPW. The changes in activity in VA were not reflected in the transcript abundance of ion transporter subunit. This could indicate regulation via other means aside from gene expression, such as phosphorylation (McDonough and Farley 1993).

IPW exposure has been shown to elevate *zo-1* in the gill (Kelly Lab observations, unpublished), however this was not observed in this experiment (Fig. 14). Fenugreek was shown to decrease transcript abundance of *zo-1* in FW-acclimated rainbow trout. In addition, *zo-1* is associated with reduced paracellular permeability of gill epithelia (Chasiotis et al. 2012b).

Changes seen in *cldn-28a,* a purported Prl-associated Cldn (Tipsmark et al. 2009), were dependent on the interaction of diet and salinity treatments. The increase was only seen in the fenugreek-IPW treatment group (Fig. 15). While the abundance of Cldn-28a has not been linked to a particular functional significance in the gill, it is reasonable to assume that a Prl-associated Cldn would serve a barrier-forming function due to the tightening of epithelia in FW, however this has not been demonstrated. The absence of change of *cldn-28a* in IPW-exposed control-fed fish is unexpected, as Prl levels are assumed to be elevated during this period. This assumption is a logical extension of the idea that Prl is elevated in SW to FW transfers, however this assumption has not been tested in this experiment or previously. Interaction dependent changes were also seen *cldn-33b*, which is likely associated with a specific non-PVC cell type in the gill (Kolosov et al. 2014). Thus, changes in *cldn-33b* may be reflective of changes in the abundance of a specific cell type.

Of the Cldns that changed as a result of varying doses of dietary fenugreek in the dose response experiment, only *cldn-5a* and *cldn-33b* exhibited similar responses with dietary fenugreek in the IPW experiment. In addition to these, *cldn-8c* decreased. The reason for this discrepancy between experiments is unknown, however in many cases, this is attributable to similar qualitative changes lacking significance. In PVC-only gill cell cultures, *cldn-8c* levels were reduced relative to heterogeneous cell culture (Kolosov et al. 2014), indicating a potential association with a non-PVC cell type. The changes observed in *cldn-5a* with dietary fenugreek mirrored the changes seen in IPW-exposed control diet fed-fish, suggesting that fenugreek alone can prompt a response that mimics hypoosmotic acclimation. Fish fed dietary fenugreek also exhibited a decrease in *cldn-1* only in IPW-exposed fish, which appears to be a restoration of transcript levels seen in FW control fish. This could either indicate a reduced requirement for *cldn*-1, possibly through an enhanced response in other Cldns, or simply a muting of the IPW response. Cldn-1 is associated with an epithelial barrier function (Inai et al. 1999)

In *cldn-10e*, *-12*, and *-30* changes in IPW treated groups were affected regardless of fenugreek treatment. It should be noted that there were no significant pairwise differences observed in *cldn-10e* and *-12*, thus indicating only minor changes. It has been shown that *cldn-10e* colocalizes with NKA-immunoreactive cells (MRCs) in *Tetraodon* (Bui and Kelly 2014), thus, the decreased transcript abundance in IPW is consistent with the decreased number of exposed MRCs as determined by SEM, but contradicts IHC analyses. As

previously discussed, *cldn-30* has barrier forming properties, particularly with respect to monovalent cations (Engelund et al. 2012). Thus the increase of *cldn-30* transcript abundance in IPW suggests a decrease in permeability. It is worth noting that no change was seen in fenugreek-fed fish in FW as observed in the dose-response experiment. The changes seen in *cldn-27b* and *cldn-32a* as a result of dietary fenugreek was only observed in IPW-exposed fish. In both cases, fish exposed to IPW had qualitatively elevated transcript levels, and this was enhanced by fenugreek. In previous studies, both Cldn transcripts have been observed to change with IPW exposure (Kelly Lab observations, unpublished), and *cldn-27b* has also been linked with barrier enhancement of epithelia (Chasiotis et al. 2012b).

In the gill, decreases were observed in *prlr* and *erβ2* in all treatments relative to the FW-control fish (Fig. 16). The changes seen in fenugreek-treated groups mirrored those observed in IPW treated groups, and there was no evidence of synergistic or additive effects, *i.e.* fenugreek alone reproduces the IPW response of both *prlr* and *erβ2*. IPW exposure had no observable effect on *gr1*, *gr2*, or *mr*. These findings are consistent with previous findings that neither IPW, nor SW has an effect on MR or GR in rainbow trout (Flores and Shrimpton 2012). However all corticosteroid receptors transcript levels decreased with dietary fenugreek. Consistent with the dose-response experiment, these findings indicate that fenugreek is linked with interaction with, or binding of corticosteroid receptors (Rosewicz et al. 1988), either directly through some component of the extract, or by promoting the release of corticosteroids.

As with the gill, *prlr* decreased in both fenugreek-treatments and IPW treatments in the hypothalamus (Fig. 17). Fenugreek and IPW acting individually both depress *prlr* levels. The depression seen in both FW-fenugreek and IPW-control is muted when both fenugreek and IPW are acting, though levels are still lower than that of the FW-control treatment group. It is an oft-assumed dogma that receptor levels are positively associated with the responsiveness of a tissue to a ligand of that receptor, thus decreased *prlr* suggests reduced responsiveness of both the gill and the hypothalamus to Prl. However, it is unknown what is causing *prlr* to change. As mentioned previously, these results contradict prior research, which has demonstrated increased *prlr* in response to IPW exposure (Flores and Shrimpton 2012). It is worth noting that a significant elevation in *prlr* was only seen after 2 days of IPW exposure (Shrimpton and Flores 2012), although abundance was 50% higher in IPW relative to FW throughout the duration. However in this study, consistent results between tissues seen lend credence to the data presented here.

A similar response to the gill is seen in *erβ2* in the hypothalamus (Fig. 17). Again, these data indicate a reduced propensity to respond to estrogen in both tissues. Estrogen levels have been shown to both increase and decrease estrogen receptors mRNA and protein, depending on the cell line (Pink and Jordan 1996). As fenugreek extract has been shown to act as an estrogen agonist (Sreeja et al. 2010), as well as increase circulating estrodiol levels (Modaresi et al. 2012), fenugreek may be modulating *erβ2* by activation, or via stimulating the release of estradiol, or via both means. The role of ERs in

osmoregulation is not well documented, however changes in expression following IPW exposure a potential link. A mirrored response in fenugreek fed fish is also an indication that fenugreek has the potential to alter the osmoregulatory capacity.

No changes were observed in *prl* or *prrp* in the pituitary and hypothalamus respectively (Fig. 18). FW transfer results in elevated [Prl] within the first 24 hours, and remains elevated for at least 3 weeks (Prunet et al. 1985), therefore the temporal expression of Prl is within the scope of this experiment with regard to IPW exposure and fenugreek treatment. Thus, the absence of a response in either *prl* or *prrp* suggests that there is no modulation on, or upstream of Prl in either IPW-exposed or fenugreek-fed fish. IPW exposure resulted in elevated levels of *prrp* and *prl* transcripts in goldfish (Kelly and Peter 2006). While the absence of changes to *prl* and *prrp* in IPW is unexpected, the effect with regard to dietary fenugreek substantiates the previous results.

4.4 Conclusions and Perspectives

It is abundantly clear that fenugreek extract has the ability to alter the osmoregulatory capacity of the rainbow trout, as evidenced by morphological and biochemical changes. What remains unclear is the mode of action. Short of changes to *prl*, *prlr*, and *prrp*, there are few genes in which changes could be associated specifically with Prl. There is more evidence that indicate alterations via the corticosteroid pathway, as supported by alterations in cortisol-associated Cldns and the near ubiquitous reduction in transcript abundance of corticosteroid receptors. Cortisol is elevated throughout lactation (Concannon et al. 1978,

Paterson and Linzell 1974). It is possible that fenugreek could result in abrogation or propagation of the corticosteroid response via agonism, or stimulation of the release of corticosteroids.

It was initially predicted that fenugreek would somehow act via Prl to produce a FW-response, at least partially. Aside from changes seen in *prlr*, there is little direct evidence that these changes can be attributed to modulations to Prl. However, in many instances, dietary fenugreek did induce a FW-like response in rainbow trout. This was seen in elevation of transcript levels in many Cldns associated with the 'barrier' function, as well as the FW NKA subunit in fenugreek-fed fish.

Mode of action aside, the ultimate goal of fenugreek treatment was to induce a FW-response, and in this regard the potential of fenugreek is promising. Given the mysterious and broad nature of the effects of fenugreek in the literature and in traditional knowledge, conducting further research with a broader and more mechanistic focus, particularly with regard to the corticosteroid pathway, would be of great interest in determining the mode of action, and therefore the usefulness of fenugreek as an aid in FW adaptation.

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6.0 APPENDIX: SKIN

In addition to the gill, the skin is also considered an osmoregulatory organ. The skin functions in all vertebrates primarily as a barrier (Chuong and Chang 2002). While sodium and chloride transport have been demonstrated in the skin, it is thought that the ion-transporting role of the skin is minor in FW compared to SW (Burgess et al. 1998, Glover et al. 2013).

In an effort to expand upon the effect of fenugreek on other osmoregulatory tissues, a sample of skin was also taken for mRNA analyses, however this was only done as part of the dose-response experiment. For complete methods on tissue sampling and RNA extraction and analyses, see sections 2.3 and 2.6.

In rainbow trout skin, there were quantifiable levels of transcripts for 16 of the 21 Cldns analyzed in the gill (Fig. 19). *cldn*-8d was present in the quantifiable range, however consistent and reliable qRT-PCR results could not be attained. Of these, seven Cldns exhibited significant changes in the abundance of the following TJ protein transcripts: *cldn-1* ($p<0.001$), *cldn-7* ($p=0.027$), *cldn-8b* ($p<0.001$), *cldn-27b* ($p<0.001$), *cldn-28a* ($p<0.001$), *cldn-29a* ($p<0.001$) and *cldn-30* ($p<0.001$) (Fig. 19).

The transcript abundance of *tric* ($p=0.015$) and *ocln* ($p=0.017$) both exhibited a significant decrease (Fig. 20). Of the scaffolding proteins, *cgn* ($p=0.03$) changed significantly however the difference was not relative to the control group (Fig. 20). *zo-1* abundance did not change.

Two TJ transcripts mirrored the changes seen in the gill, *cldn-7* and *cldn-*

30, where both transcripts were elevated in fenugreek-treated groups. As in the gill, these were most abundant Cldn transcripts in the skin. In the gill, these Cldns have been associated with decreased paracellular permeability (Kelly and Chasiotis 2011). Gill *cldn-27b* is affected by environmental pH (Kumai et al. 2011). In the gill, *cldn-8b, 27b,* and *29a* are associated with the development of the resistive properties of epithelia in cell culture (Kolosov et al. 2014), which indicates that these Cldns are barrier-forming. Changes seen in these Cldns suggest a potential decrease in paracellular permeability in the bicellular TJ.

There was a significant change in *cldn-28a*, however this difference was not relative to the control treatment, thus it is difficult to make inferences regarding the changes. *cldn-28a* levels are positively associated with [Prl] (Tipsmark et al. 2009). While *cldn-28a* was the third most abundant Cldn in the gill, it was the second least abundant of the Cldns quantified in the skin.

Tric knockdown results in a dramatic decrease in the resistive properties in cultured rainbow trout gill epithelia (Kolosov and Kelly 2013), and *tric* abundance is elevated in the intestine following seawater transfer (Tipsmark and Madsen 2012). In the stenohaline goldfish, *ocln* abundance was elevated in IPW, and was associated with decreased permeability with cortisol exposure (Chasiotis et al. 2009, Chasiotis et al. 2010). Thus a decrease in *tric* and *ocln* abundance may lead to increased epithelial permeability.

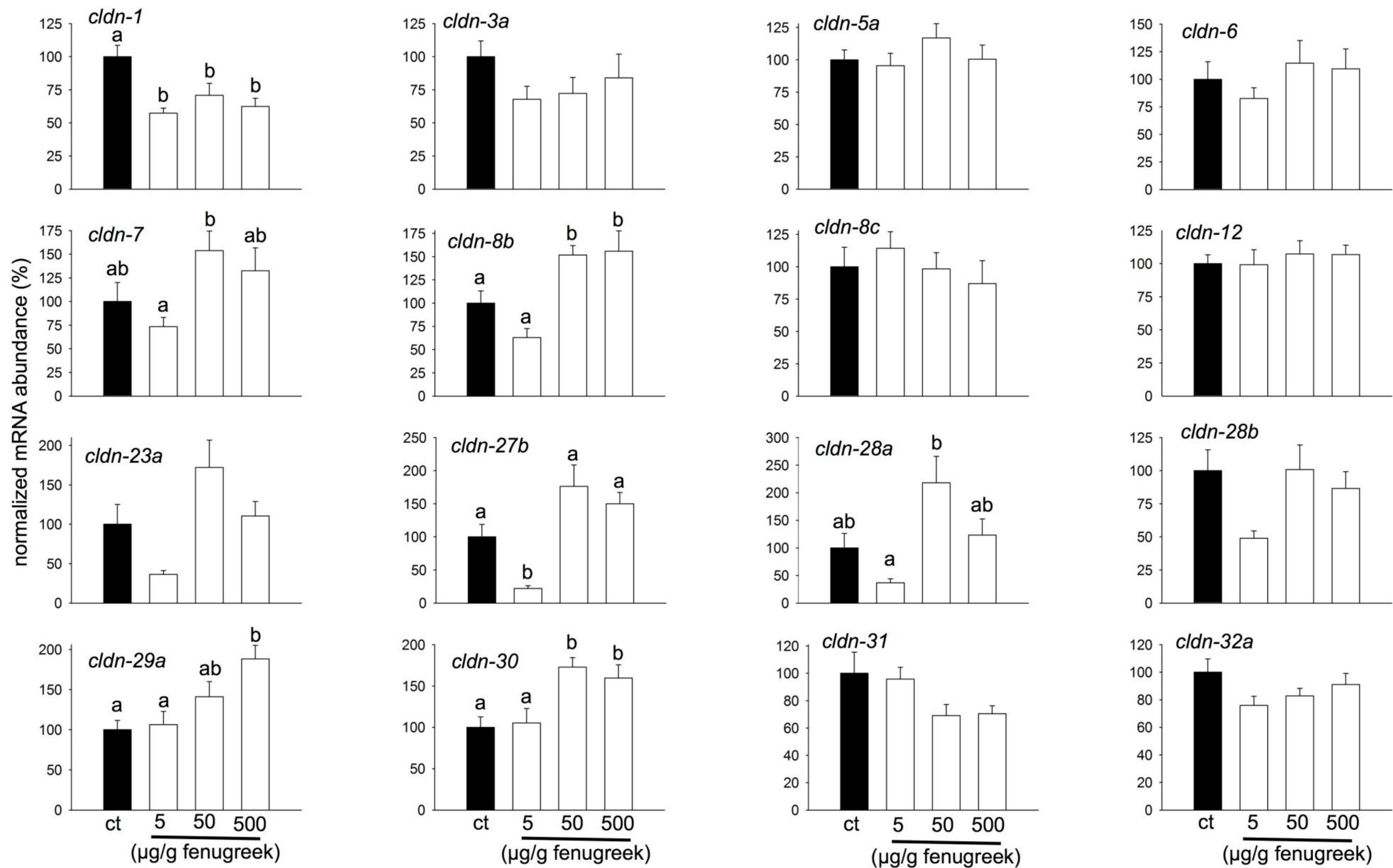

Figure 19: Effect of various dietary levels of fenugreek (5, 50, 500 μg fenugreek/g diet) on relative transcript abundance of the claudin (*cldn*) of tight junction (TJ) proteins in the skin. A diet without fenugreek, but otherwise treated identically to experimental diets was designated as control (ct). Transcript abundance was normalized to β-actin as a reference gene. Transcript abundance was expressed relative to ct fish. Values are expressed as mean +/- SE (n=10).

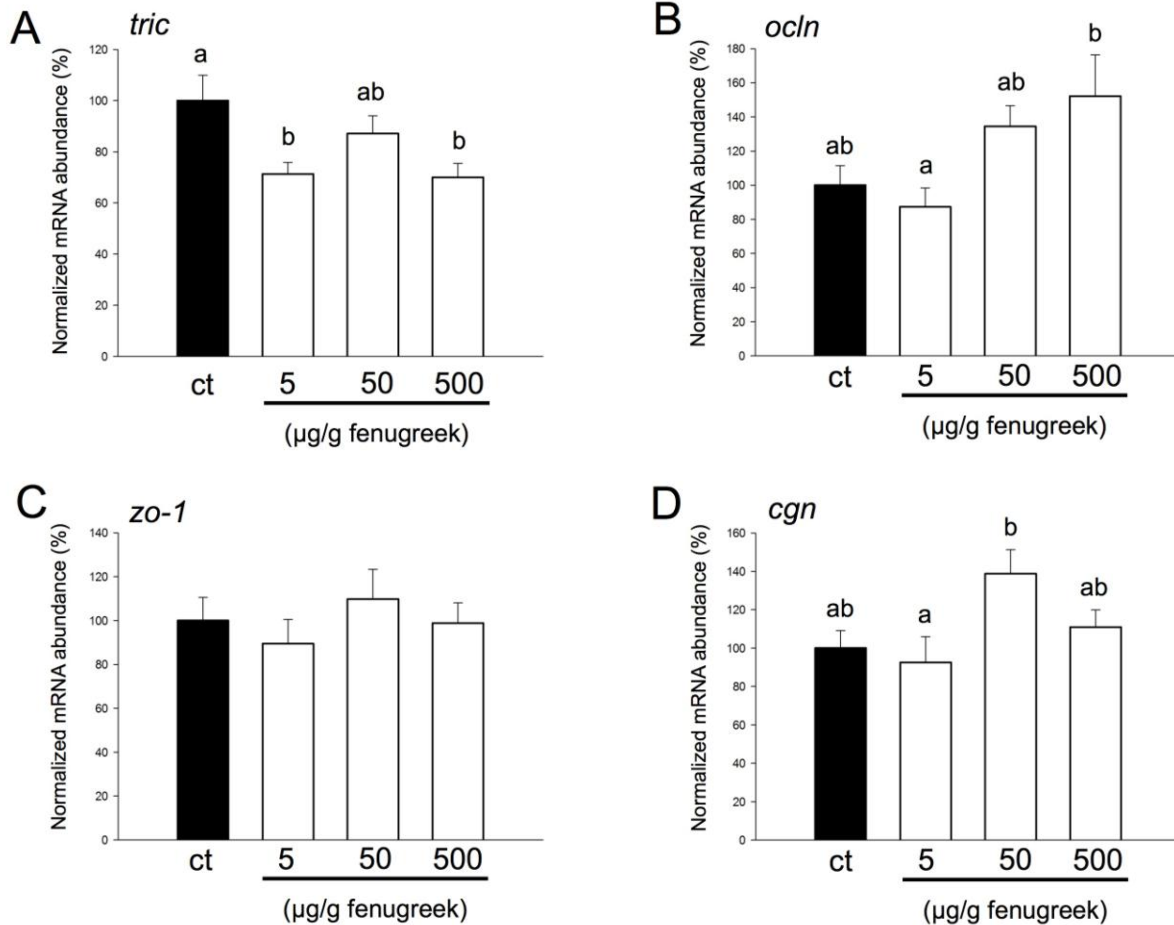

Figure 20: Effect of fenugreek on the relative abundance of (A-C) scaffolding proteins and (D) *tric* in rainbow trout skin. Transcript abundance was normalized to β-actin and expressed in fenugreek treatments relative to the control diet (0µg/g) fed group, which was assigned a value of 100%. Significant differences were found in (A) *cgn* (p=0.03), (B) *ocln* (p=0.017), (D) *tric* (p=0.015). Data are expressed as mean +/- SE (n=10).