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Hormonal effects on expression of osmoregulatory genes in the gill of Japanese medaka (Oryzias latipes)

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Hormonal effects on expression of osmoregulatory genes in the gill of Japanese medaka (*Oryzias latipes*)

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By

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1. Abstract

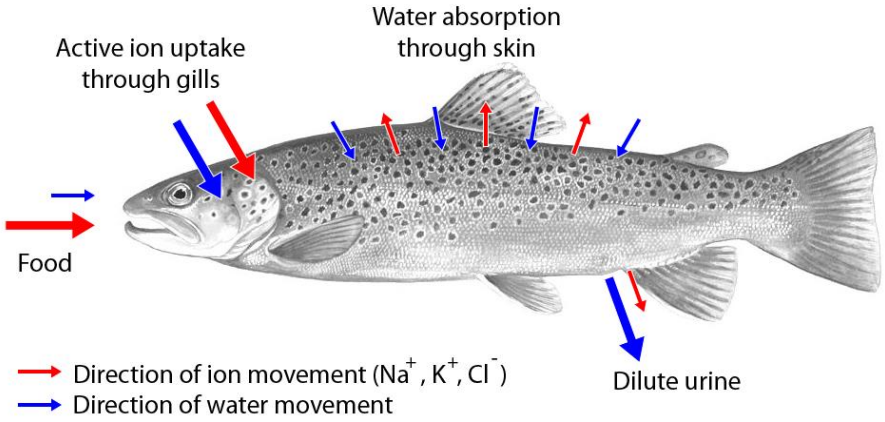
Japanese medaka (*Oryzias latipes*) are euryhaline teleost and evolved divergently from the well-studied model organism zebrafish (*Danio rerio*). Claudins (cldns) are tight junction proteins known to regulate the permeability of the paracellular space, but little is known about the influence of the endocrine system on their regulation. In this study, *in vitro* gill culture model was used to test the effect of three osmoregulatory hormones (cortisol, growth hormone, and prolactin) on eight cldns previously identified in the gills of medaka. The stress hormone cortisol generally up-regulated the genes studied from 1.2- to 5-fold at the exceptions of *cldn-28a* and *-28b*. Growth hormone had little effect and only increased the transcription of *cldn-10f* 2.5-fold, while prolactin up-regulated the expression of *cldn-28b*, *-10c*, and *-10f*, to 2-, 2.5-, and 2-fold respectively. The results support the hypothesis that *cldns-27a*, *-28a*, *-28b*, and *-30c* are barrier-forming cldns, with *-28b* being induced by FW conditions. Further experiments need to be performed before the hormonal regulation of *cldn-10* isoforms can be fully understood. The *in vitro* model shown here is a useful tool to resolve endocrine control of gill proteins involved in osmoregulation. Further investigation of the possible interactions of cortisol and prolactin or growth hormone would improve the understanding of claudin regulation by hormones.

2. Introduction

All living things must maintain a near constant internal salt concentration, or osmolarity, in order to survive. Aquatic environments present a unique challenge to the fish that live in them as salinity exerts an abiotic pressure on the organism. Teleost fish typically maintain an internal salinity of 1.1%. When a fish is in fresh water (FW), the external salinity is less than 0.05%, so the fish needs to actively pump ions in from the environment and attempt to prevent water from entering by osmosis (Edwards and Marshall 2013). The fish does this by pumping ions in across the gill epithelia, absorbing ions from food in the intestine, and producing copious amounts of dilute urine to expunge excess water (Figure 1). In seawater (SW), the external salinity is around 3.4%, so a fish needs to excrete excess ions and ingest water to compensate for the loss of water by osmosis (Edwards and Marshall 2013). The fish does this by actively drinking seawater and absorbing the water in the intestine, pumping ions out across the gills epithelia, and expunging divalent ions in their scanty isotonic urine.

Some fishes are only able to survive in either FW or SW, they are called stenohaline (Marshall and Grosell 2006). Few fish species are euryhaline, meaning they are able to survive in a wide range of salinities. This ability has evolved independently in several teleost lineages including salmonids, tilapia, and sea bass (Schultz and McCormick 2013). Some species, like Mozambique Tilapia (*Oreochromis mossambicus*) are especially well noted for their wide range of tolerance from FW to water with salinity greater than two-fold ordinary SW (Suresh and Lin 1992). Japanese medaka (*Oryzias latipes*) are of particular interest in addition to their euryhalinity, because of their

Water and salt exchange in FW fishes



Water and salt exchange in SW fishes

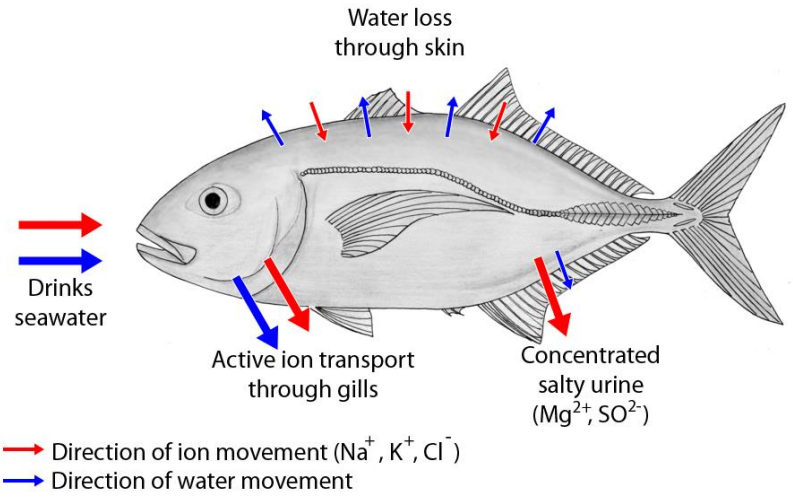


Figure 1. Model of ion regulation in FW and SW fishes
FW fish actively pump ions in and need to expunge excess water that flows in by osmosis. They do this by consuming ions in the form of food and pumping them in across the intestinal epithelia, pumping ions in from the water across the epithelia of the gill, and produce copious amounts of very dilute urine. SW fish actively pump ions in and counteract the loss of water that is caused by osmosis. They do this by actively drinking seawater to absorb the water in their intestines, expunge ions in a isotonic urine, and pump ions out across the gill epithelia. Thicker arrows are indicitive of greater fluxes in movement, compared to thin arrows.

divergent evolution from the well-studied stenohaline model, zebrafish (*Danio rerio*). Japanese medaka are native to Southeast Asia and are often found in rice patties where salinity fluctuates with the tide. They are good model organisms because they have a small genome of 800 Mbp (Ishikawa 2000; Tanaka 1995) that has been entirely sequenced (Kasahara et al. 2007) and therefore makes genomic analysis easier to perform.

In both stenohaline and euryhaline fishes the gill is the main osmoregulatory organ (Edwards and Marshall 2013). Ionocytes, also called mitochondria-rich cells, are specialized cells that primarily transport ions across the epithelia, including the gill (Evans et al. 2005). The main pump in both FW and SW ionocytes is a Na^+/K^+-ATPase (NKA), which has been studied recently in medaka (Bollinger et. al. pers. communication) and in several other teleost species (Tipsmark and Madsen 2009; Breves et al. 2014). This protein, coupled with other channels and co-transporters, directs ions through the cells and across the epithelia. A model for ion transport in Japanese medaka for both FW and SW ionocytes has been previously proposed (Hsu et al. 2014). A simplified version shown in Figure 2 displays the salinity-specific expression of two genes, Cystic Fibrosis Transmembrane conductance Regulator chloride channel (CFTR) and Na^+/Cl^--co-transporter (NCC-like2).

The expression of ion transporters in ionocytes is primarily regulated by the endocrine system. Prolactin is primarily responsible for FW acclimation, while growth hormone (GH) acts to up-regulate ion secretion in SW environments (Takei and McCormick 2013). Cortisol improves the ability of fish to osmoregulate in both FW and SW; it also interacts with both GH and prolactin (Takei and McCormick 2013). However,

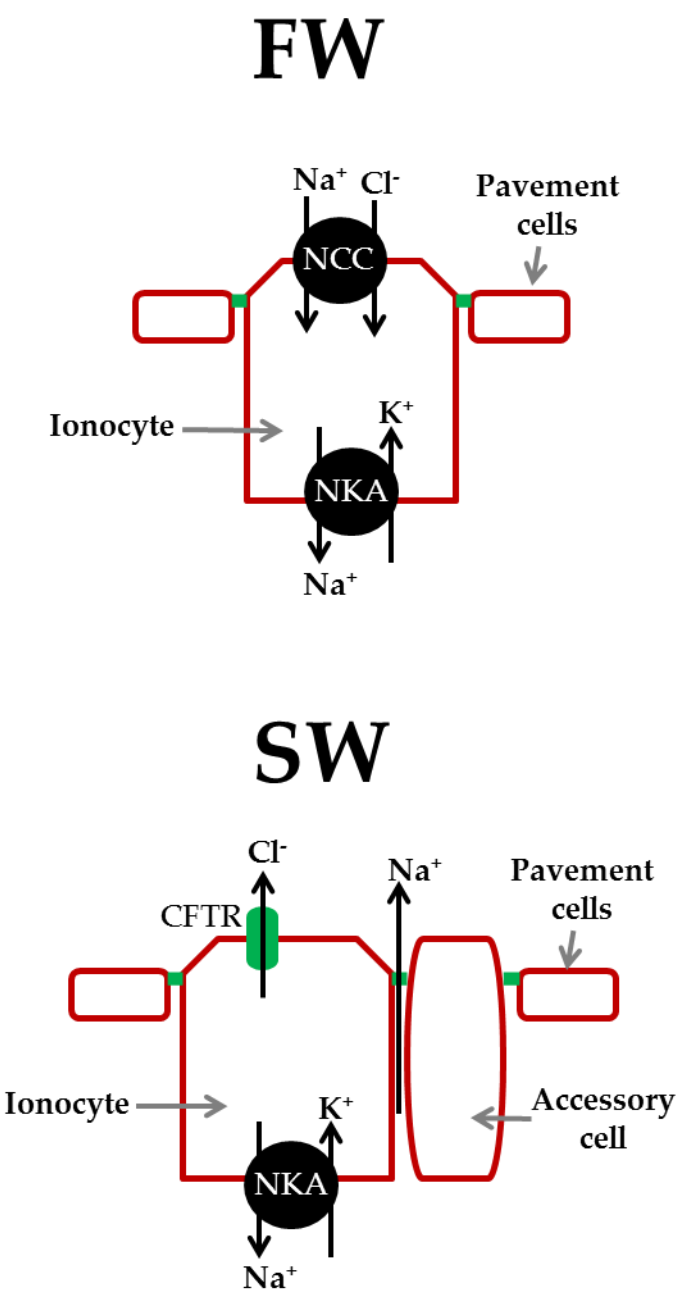


Figure 2. Simplified model of medaka ionocyte in FW and SW
CFTR is a protein found exclusively in SW ionocytes, while NCC is found primarily in FW ionocytes. CFTR: Cystic Fibrosis Transmembrane Conductance Regulator chloride channel; NCC: Na^+,Cl^--cotransporter; NKA: Na^+/K^+-ATPase.

these hormones and their effect on protein expressions in ionocytes have not been previously tested in Japanese medaka.

In between all epithelial cells, including those found in the gill, tight junctions regulate the permeability of the tissue (Furuse 2010). Among tight junction proteins, the superfamily of claudins (cldns) have been shown to be the proteins which form either a barrier or an ion-selective pore (Furuse 2010). Cldns are 22-27 kDa proteins with four transmembrane domains, N- and C-terminus in cytoplasm, and two extracellular loops (ECLs; Furuse 2010). A specific domain within the first ECL is responsible for the ion selectivity of the cldn isoforms (Furuse 2010).

While 27 cldn genes have been described in mammals (Günzel and Yu 2013), genome duplication and tandem gene duplication events are at the origin of an expanded cldn family in teleost including 54-56 different cldn genes (Loh et al. 2004; Baltzegar et al. 2013). In Japanese medaka 13 cldn isoforms have been identified in osmoregulatory organs, including eight primarily localized in the gill: cldn-10c, -10d, -10e, -10f, -27a, -28a, -28b, and -30c (Bossus et al. pers. communication). In a recent study in our laboratory, it was shown that all four gill *cldn-10* isoforms are up-regulated in SW, *cldn-28b* is up-regulated in FW, and the expression of other branchial *cldns* are maintained at a near constant level independent of salinity. However no information is currently available on their hormonal regulation.

In the current study we therefore investigated the effect of osmoregulatory hormones on gill cldn expression in the Japanese medaka using *in vitro* incubations of gill samples in controlled media with or without the selected hormones. Both *cftr* and *ncc-like2* were also measured, used respectively as SW- and FW-specific indicators, as

mentioned earlier they are highly expressed in either environment. *In vitro* experiments allow for tighter control of what conditions the gills are exposed to, since the fish's natural endocrine system is no longer a factor. Also more experimental conditions can be performed with fewer animals.

3. Materials and Methods

3.1 Animals

Adult Japanese medaka were obtained from Pentair Aquatic Ecosystems, Inc., (Florida, USA). They were kept in 450 L tanks filled with aerated de-chlorinated tap water, mechanically and biologically filtered (0.34 mM Na^+, 0.64 mM Ca^{2+}, 0.09 mM Mg^{2+}, 0.03 mM K^+). They were kept under a 14 hrs light/10 hrs dark photoperiod at 20°C. The fish were fed once daily with frozen brine shrimp (San Francisco Bay Brand, Inc., Newark, CA, USA). Food was withheld from the fish on sampling days. All experiments and handling procedures were approved by the Animal Care and Use Committee of the University of Arkansas (IACUC protocol numbers 14042 and 11005).

3.2 Experiments and Sampling

Both ovine prolactin and ovine GH were purchased from UCLA Research and Education Institute (CA, USA). The cortisol used was Hydrocortisone 21-Hemisuccinate Sodium salt (Sigma-Aldrich, Co., St Louis, MO, USA). The fish were quickly killed by puncturing their brain with a scalpel. The gill was removed, individual arches separated, then immediately place in Dulbecco's Modification of Eagle's Medium (DMEM; Cellgro by Corning, manufactured by Mediatech, Inc., Manassas, VA, USA) for one hour before

transfer into experimental conditions. A total of 16 fish were sacrificed in this manner for each set of experiments.

One gill arch from two different fish were both placed under each different condition to provide enough tissue to perform RNA extraction, having thus one biological replicate for each condition. At that time, one group was placed in TRI Reagent® (Sigma-Aldrich) as a preincubation control. The other experimental groups had an incubation period of 16 hours. Three sets of experiments were performed:

1. A cortisol concentration response experiment where the groups included the preincubation control, an incubation control, and DMEM with 0.1 μg/mL, 1 μg/mL, and 10 μg/mL of cortisol with N=8.

2. The same experiment was repeated with ovine GH and at concentrations of 0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL each with N=6.

3. The same experiment was repeated with ovine prolactin at concentrations of 0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL each with N=6.

Immediately after the incubation period the samples were placed in 250 μL of Tri Reagent® and homogenized as described below.

3.3 RNA isolation, cDNA synthesis, and quantitative PCR (qPCR)

Tissues were homogenized in Tri Reagent® using a VWR PowerMax 200 rotating knife homogenizer (Advanced Homogenizing System, Manufactured by Pro Scientific for Henry Troemner LLC, Thorofare, NJ, USA). Total RNA was extracted according to the manufacturer's protocol. The RNA pellet was dissolved into nuclease-free water, the concentration and purity (A260/A280 ratio at least >1.80) were estimated using a

NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). For each sample, first strand cDNA was obtained from 1 µg of total RNA using the High Capacity cDNA reverse transcription kit from Applied Biosystems (Foster City, CA, USA) in a final volume of 20 µL and following the manufacturer's protocol.

All primers used in this study were previously made and optimized in recent work from our laboratory (Bossus et al. pers. communication; Bollinger et al. pers. communication). Quantitative real-time PCR (qPCR) analyses were performed using BioRad CFX96 platform thermocycler (BioRad, Hercules, CA, USA), the SYBR® Green JumpStar™ Taq ReadyMix™ (Sigma-Aldrich) and a primer concentration of 150 nM each, in a final volume of 15 µL. The qPCR were performed with an initial heating step at 94°C for 2 min, followed by 40 cycles of a denaturation step at 94°C for 30 sec and an annealing/elongation step at 60°C for 1 min. Melting curve analysis was performed from 55 to 94°C with a gradient of 5 s per °C to confirm the specificity of the reaction. Standard curves were used to determine the primer sets efficiency. Relative mRNA expression was calculated according to Pfaffl's equation (Pfaffl 2001): $C_n=(1+E_a)^{-Ct}$, with C_n the relative copy number, Ct the threshold cycle of the target gene, and E_a the amplification efficiency.

To normalize all expression data, geometric means were used, calculated from the corrected expression data of two or three normalization genes using GeNorm software (Biogazelle, Zwijnaarde, Belgium). The genes used were *ef1α* and *s18* for the cortisol concentration response and *ef1α*, *s18*, and *rpl7* for the prolactin and GH concentration response experiments. There was no significant variation observed for any normalization

gene (data not shown). Primer-dimer association was controlled with no template controls (NTC).

3.4 Statistics

All statistical analyses were performed using GraphPad Prism 5.0 software (San Diego, CA, USA). The homogeneity of variances was tested with Bartlett's test. To meet the ANOVA assumption of homogeneity of variances, logarithmic transformation of data was done when required. Bonferroni's post-test was used to compare the control to the preincubation and to the hormone incubations. Results are expressed as the mean ± standard error mean (s.e.m.) with a p-value of $p<0.05$.

4. Results

4.1 Cortisol concentration response experiment

The *cftr* expression was significantly increased after overnight *in vitro* incubation at 10 μg/mL of cortisol (Figure 3A). The *ncc-like2* expression was also increased at both 1 μg/mL and 10 μg/mL of cortisol (Figure 3B). All cldn isoforms except *cldn-27a* had a significant drop in expression when comparing initial sampling control (preincubation) with overnight *in vitro* incubation control groups. Cortisol significantly up-regulated the expression of all *cldn-10* isoforms, as well as *cldn-27a* at 10 μg/mL, and *cldn-30c* at 0.1 μg/mL (Figure 4).

4.2 Prolactin concentration response experiment

In this experiment *ncc-like2 in vitro* control group was significantly decreased compared to preincubation initial levels and it was also the case for *cldn-10d, -10f, -28a, -28b* and -*30c* (Figure 5A and 6). However, *cftr* expression did not change after overnight *in vitro* treatment (Figure 5B). Prolactin significantly inhibited the expression of *cftr* at 1 μg/mL

and up-regulated *ncc-like2* at both 0.1 μg/mL and 1 μg/mL compared to the control (Figure 5). Prolactin significantly increased the expression of *cldn-10d, -10f,* and *-28b* at 1 μg/mL and also *-28b* at 0.1 μg/mL (Figure 6).

4.3 Growth hormone concentration response

The *ncc-like2,* c*ldn-10d, -10f, -28b* and *-30c* expression all significantly decreased after overnight *in vitro* incubation when compared to the initial sampling values, while *cftr* was unchanged (Figure 7 and 8). GH significantly increased the expression of *cldn-10f* at a concentration of 0.1 μg/mL but there were no significant effects of this hormone on any other target genes (Figure 8).

5. Discussion

Japanese medaka have only been used for a few years as an alternative model to the stenohaline *D. rerio* for studies on trans- and paracellular osmoregulatory mechanisms (Hsu et al. 2014). The super family of tight junction proteins called cldns are known to be responsible for the regulation of the paracellular pathway by creating either a barrier in FW gill or a "leaky" epithelia in SW gill (Chasiotis et al. 2012). Eight cldn isoforms were recently identified in the medaka gill (Bossus et al. pers. communication) and therefore their hormonal regulation was analyzed in this present work. The stress hormone of cortisol generally up-regulated the genes studied with the exceptions of *cldn-28a* and *-28b*. GH had little effect and only increased the transcription of *cldn-10f,* while prolactin up-regulated the expression of *ncc-like2, cldn-28b, -10c,* and *-10f.*

5.1 Constitutive claudins in the medaka gill

Previously *cldn-27a, -28a,* and *-30c* expression in medaka were shown to be unaffected by variation in salinity, suggesting an equal constitutive role in FW and SW (Bossus et al. pers. communication). Even though the SW epithelia is considered more "leaky", it is still desirable to have a majority of the tight junctions serve as a barrier. According to the model of ion secretion in the gill shown in Figure 2, the electrochemical gradient that draws Na^+ out is only present when accessory cells are present next to ionocytes. However the majority of the gill epithelia is composed of pavement cells with a high transepithelial resistance, creating a barrier. The accessory cell-ionocyte structures, which create a pore permitting the entry of Na^+, need to be sparse to avoid a detrimental effect on ionic balance. Thus barrier Cldns are needed irrespective of environmental salinity.

No effect of the FW acclimating prolactin or the SW acclimating GH was observed for *cldn-27a, -28a,* and *-30c,* which is in accordance with their constitutive expression in both FW and SW. The results of these experiments agree with the previous findings as these cldns were not affected by either salinity specific hormone. Furthermore, in a previous study performed *in vivo* using Atlantic Salmon (*Salmo salar*), the findings were similar with the only exception being *cldn-28a* expression was increased by prolactin (Tipsmark et al. 2009).

Cortisol was shown in this study to increase the expression of both *cldn-27a* and *-30c,* but did not influence *cldn-28a*. This elevation could be to prevent any fluxes of ions or water during short term salinity stress. The same effect was observed *in vitro* with *S. salar,* however these two cldn isoforms were found to be FW specific in that organism (Tipsmark et al. 2009). It is notable to mention that *cldn-30c* expression increased at 0.1

μg/mL of cortisol in medaka, instead of 10 μg/mL as the other targets. This result might indicate a differential regulation of this gene which would react at a lower cortisol level than the other genes.

Only the expression of one isoform, *cldn-28a*, was not influenced by salinity or hormonal treatments. This constitutive cldn when observed *in vivo* would likely be expressed at the same level regardless of the external conditions. It could be found deeper in the epithelia as a structural protein. In *S. salar* this isoform is also constitutively expressed across salinity but is surprisingly up-regulated in the presence of prolactin, which had not yet been resolved (Tipsmark et al. 2009).

5.2 Fresh water claudins in the medaka gill

The expression of *ncc-like2* was used as an indicator of the presence of FW ionocytes. This gene was up-regulated by both cortisol and prolactin at higher concentrations, which indicates that the prolactin induced the formation of FW ionocytes. Prolactin also decreased the expression of *cftr*, which indicates an antagonistic effect on SW ionocytes. Prolactin has previously been shown to up-regulate the expression of *ncc* in zebrafish and Nile tilapia (Breves et al. 2013; Breves et al. 2014).

In a recent study from our laboratory, *cldn-28b* was the only isoform in the gill of medaka found to be up-regulated in FW (Bossus et al. pers. communication). The only hormone that affected the expression of this isoform is prolactin, furthering the support for a FW-specific cldn. This putative barrier cldn might be found in the tight junctions next to ionocytes where they could replace cldn-10s in the SW to FW transition, sealing leaky tight junctions between ionocytes (Kwong et al. 2012). This could be tested in the future with immunohistochemistry.

5.3 Seawater claudins in medaka gills

To test for the formation of SW ionocytes, the response of *cftr* to GH was measured, but no up-regulation was observed. Another indicator of the effect of GH is the expression of insulin-like growth factor (IGF1), which has been found in several other species to increase in the presence of GH (Evans 2002, Pierce et al. 2011). The expression of IGF1 was measured for the GH concentration response samples and no significant effect was observed (data not shown). This result may suggest that the ovine GH did not reach and/or activate the GH receptor in the present *in vitro* medaka system. In future a GH more homologous to that found in medaka could be used.

Of all the SW cldn isoforms, only *cldn-10f* was up-regulated by GH while it was also up-regulated by prolactin and cortisol. This suggests a pathway that is activated by both the GH and prolactin receptors in the gill. The expression of *cldn-10c* was also increased by both prolactin and cortisol. The role of those putative pore-forming cldns in FW is currently unknown. The increase in expression from prolactin follows the same pattern as *ncc-like2*, suggesting that they are possibly co-localized, which could be tested in the future with immunohistochemistry. Finally, *cldn-10d* and *-10e* were up-regulated by cortisol. This effect may represent the underpinning of the up-regulation of these *cldns* in SW (Bossus et al. pers. communication), as this hormone is known to increase during SW acclimation to possibly assist in the stressful transition from FW to SW.

5.4 Conclusion and Future work

In conclusion, the *in vitro* culture model that was used in these experiments has a strong potential for the future with its simple design and easily controllable conditions. To gain a more complete understanding of hormonal regulation in the gills of medaka further

experiments must be done. These may include a GH concentration response with an teleost fish GH ortholog to ascertain a specific action in medaka. In addition, experiments testing the interaction of cortisol with GH or prolactin can be done, along with time course experiments to evaluate the speed at which changes in expression occur. There is still much to explore with the hormonal regulation of cldns and these studies provide new knowledge on cortisol and prolactin regulation of gill function. Furthermore they will help ask new questions and give guidance in the direction of future studies.

6. Appendix

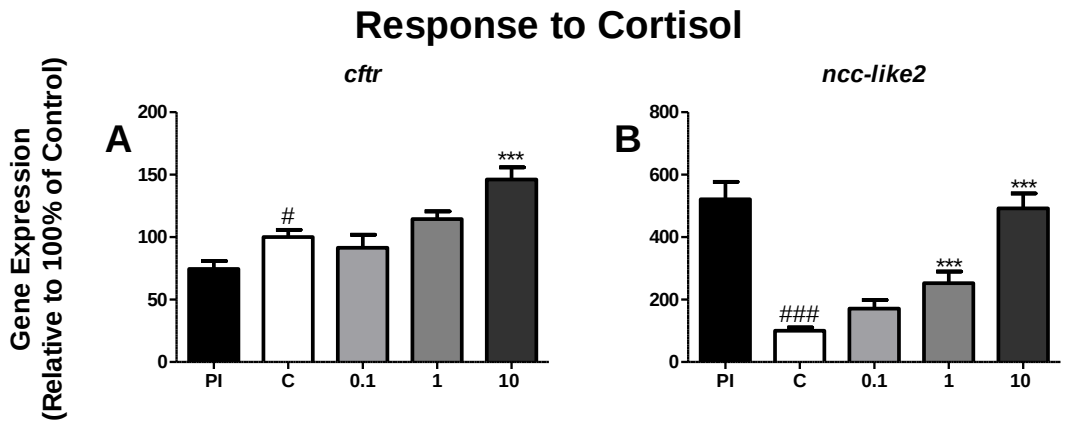


Figure 3. Response of *ncc-like2* (A) and *cftr* (B) expression to cortisol exposure. Results are expressed with mean ± s.e.m. (N=8) relative to 100% of control expression. PI: preincubation control, C: control, numbers are indicative of the concentration of cortisol in μg/mL. Pound signs (#) are indicative of significance of the preincubation from the overnight incubation control, while asterisks (*) are indicative of significance of the concentration response from control. One symbol represents $p<0.05$ and three represents $p<0.001$.

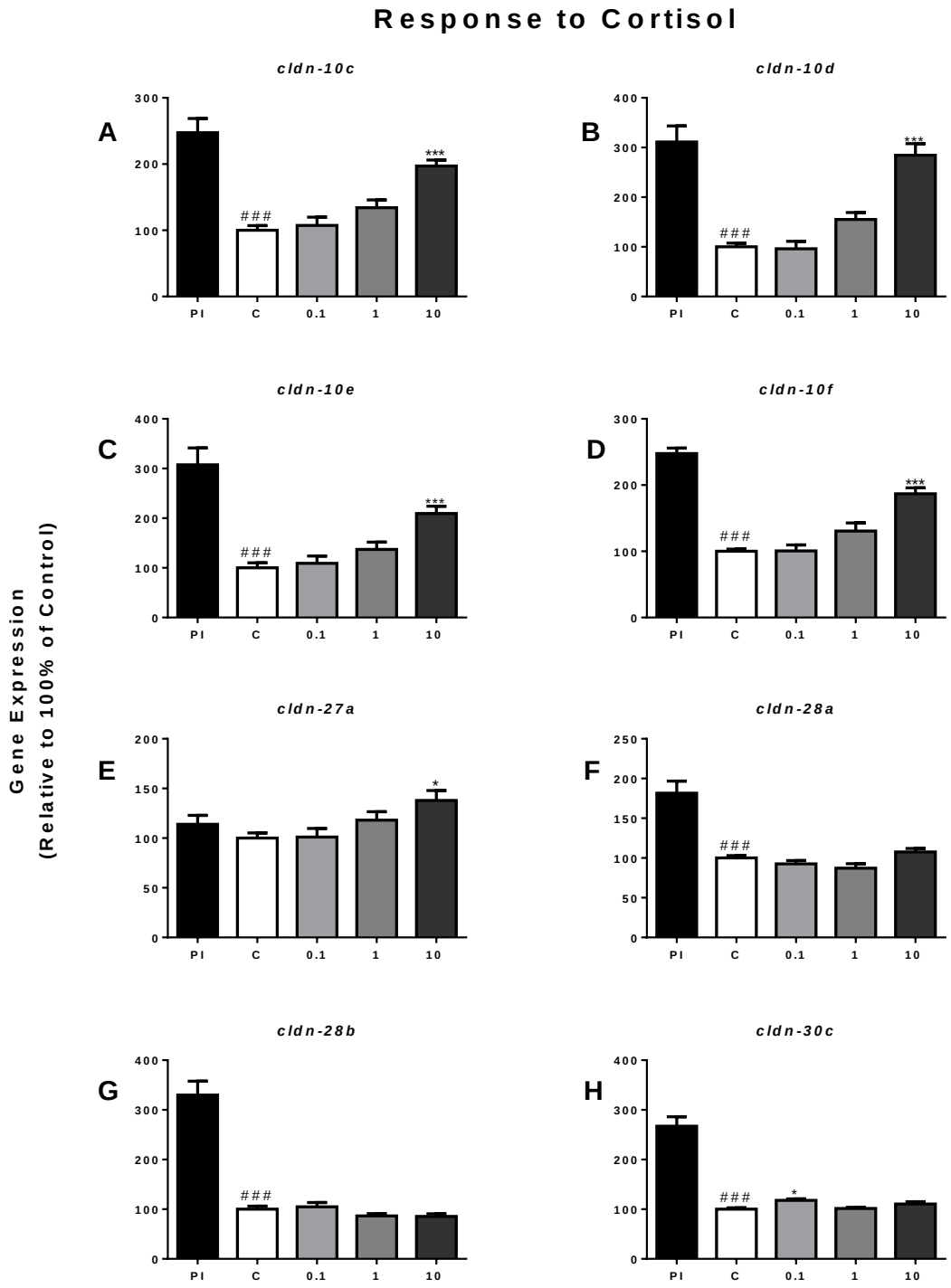

Response to Cortisol
Gene Expression
(Relative to 100% of Control)
cldn-10c
cldn-10d
cldn-10e
cldn-10f
cldn-27a
cldn-28a
cldn-28b
cldn-30c
A
B
C
D
E
F
G
H
PI
C
0.1
1
10
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Figure 4 (previous page). Response of *cldns-10c* (A), *-10d* (B), *-10e* (C), *-10f* (D), *-27a* (E), *-28a* (F), *-28b* (G), and *-30c* (H) expression to cortisol exposure. Results are expressed with mean ± s.e.m. (N=8) relative 100% of the control expression. PI: preincubation control, C: control, numbers are indicative of the concentration of cortisol in μg/mL. Pound signs (#) are indicative of significance of the preincubation from the overnight incubation control, while asterisks (*) are indicative of significance of the concentration response from control. One symbol represents $p<0.05$ and three represents $p<0.001$.

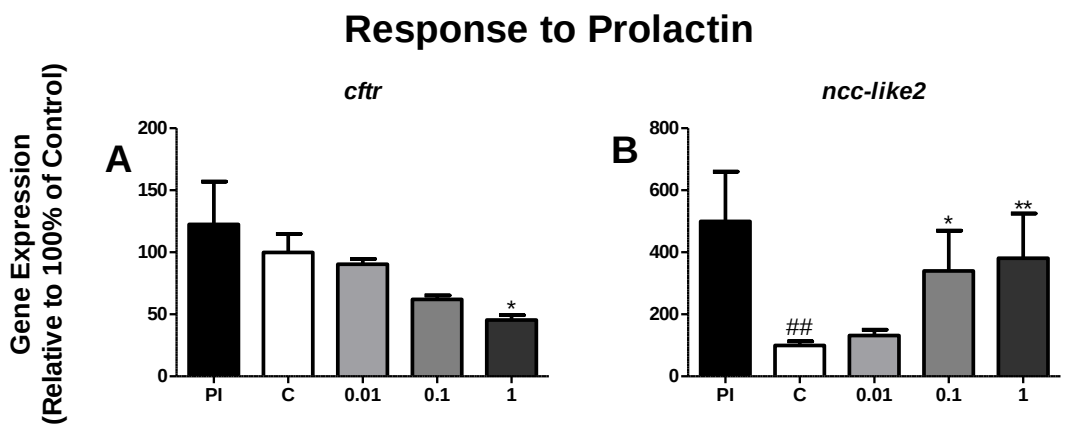

Figure 5. Response of *ncc-like2* (A) and *cftr* (B) expression to prolactin exposure. Results are expressed with mean ± s.e.m. (N=6) relative to 100% of control expression. PI: preincubation control, C: control, numbers are indicative of the concentration of cortisol in μg/mL. Pound signs (#) are indicative of significance of the preincubation from the overnight incubation control, while asterisks (*) are indicative of significance of the concentration response from control. One symbol represents $p<0.05$ and two represents $p<0.01$

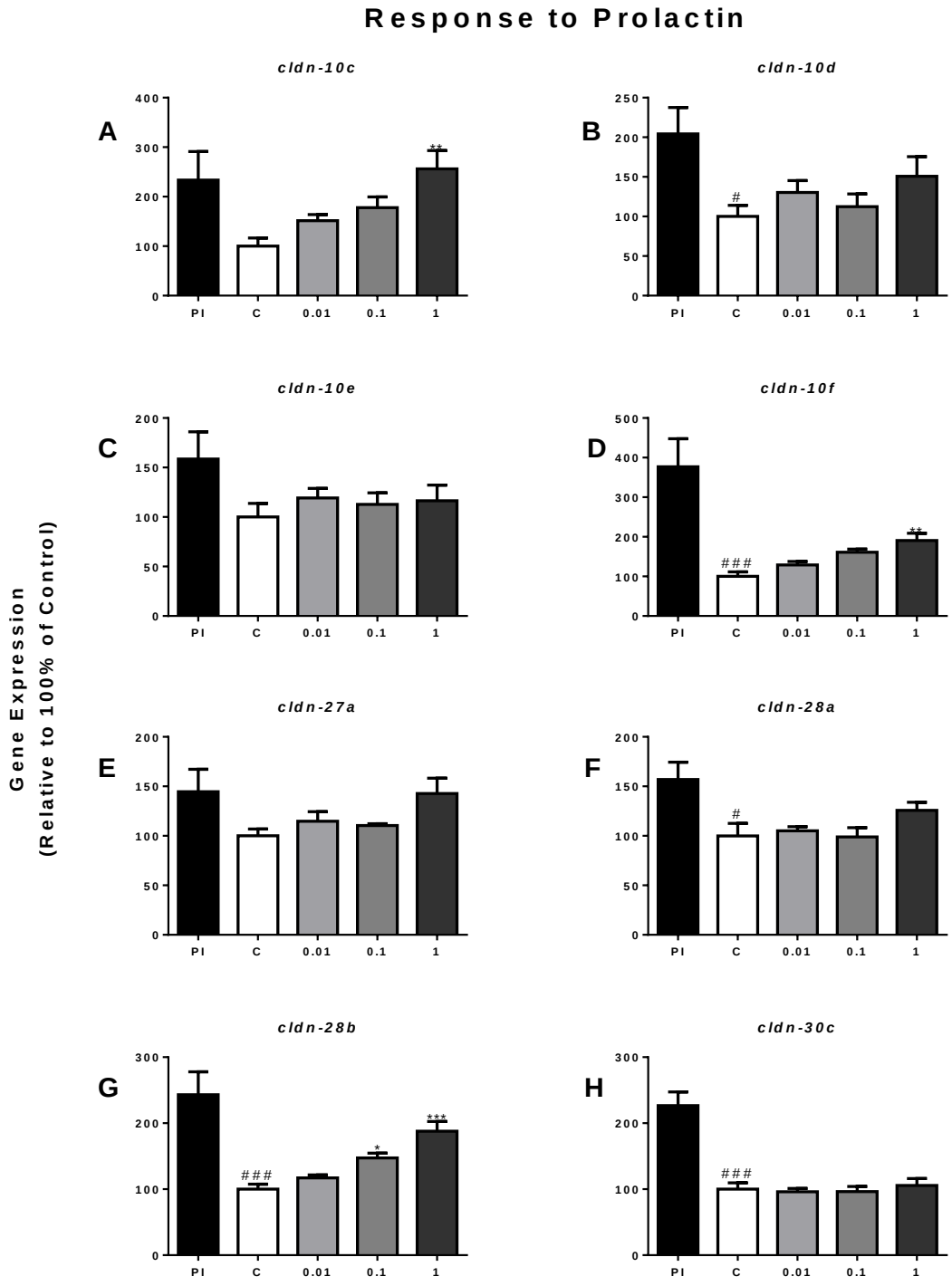
Response to Prolactin
Gene Expression
(Relative to 100% of Control)
A
cldn-10c
B
cldn-10d
C
cldn-10e
D
cldn-10f
E
cldn-27a
F
cldn-28a
G
cldn-28b
H
cldn-30c
PI
C
0.01
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Figure 6 (previous page). Response of *cldn-10c* (A), -*10d* (B), -*10e* (C), -*10f* (D), -*27a* (E), -*28a* (F), -*28b* (G), and -*30c* (H) expression to prolactin exposure. Results are expressed with mean ± s.e.m. (N=6) relative to 100% of control expression. PI: preincubation control, C: control, numbers are indicative of the concentration of cortisol in μg/mL. Pound signs (#) are indicative of significance of the preincubation from the overnight incubation control, while asterisks (*) are indicative of significance of the concentration response from control. One symbol represents $p<0.05$, two represents $p<0.01$, and three represents $p<0.001$.

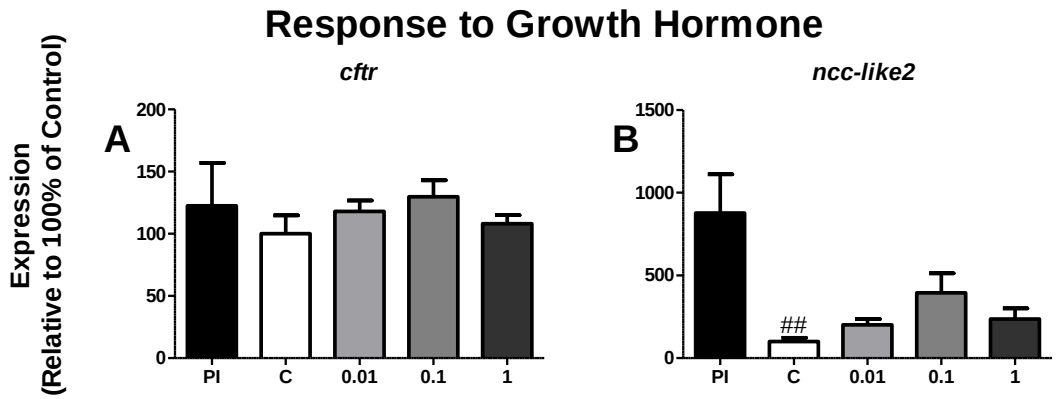

Figure 7. Response of *ncc-like2* (A) and *cftr* (B) expression to growth hormone exposure. Results expressed with mean ± s.e.m. (N=6) relative to 100% of control expression. PI: preincubation control, C: control, numbers are indicative of the concentration of cortisol in μg/mL. Pound signs (#) are indicative of significance of the preincubation from the overnight incubation control, while asterisks (*) are indicative of significance of the concentration response from control. Two symbols represents $p<0.01$.

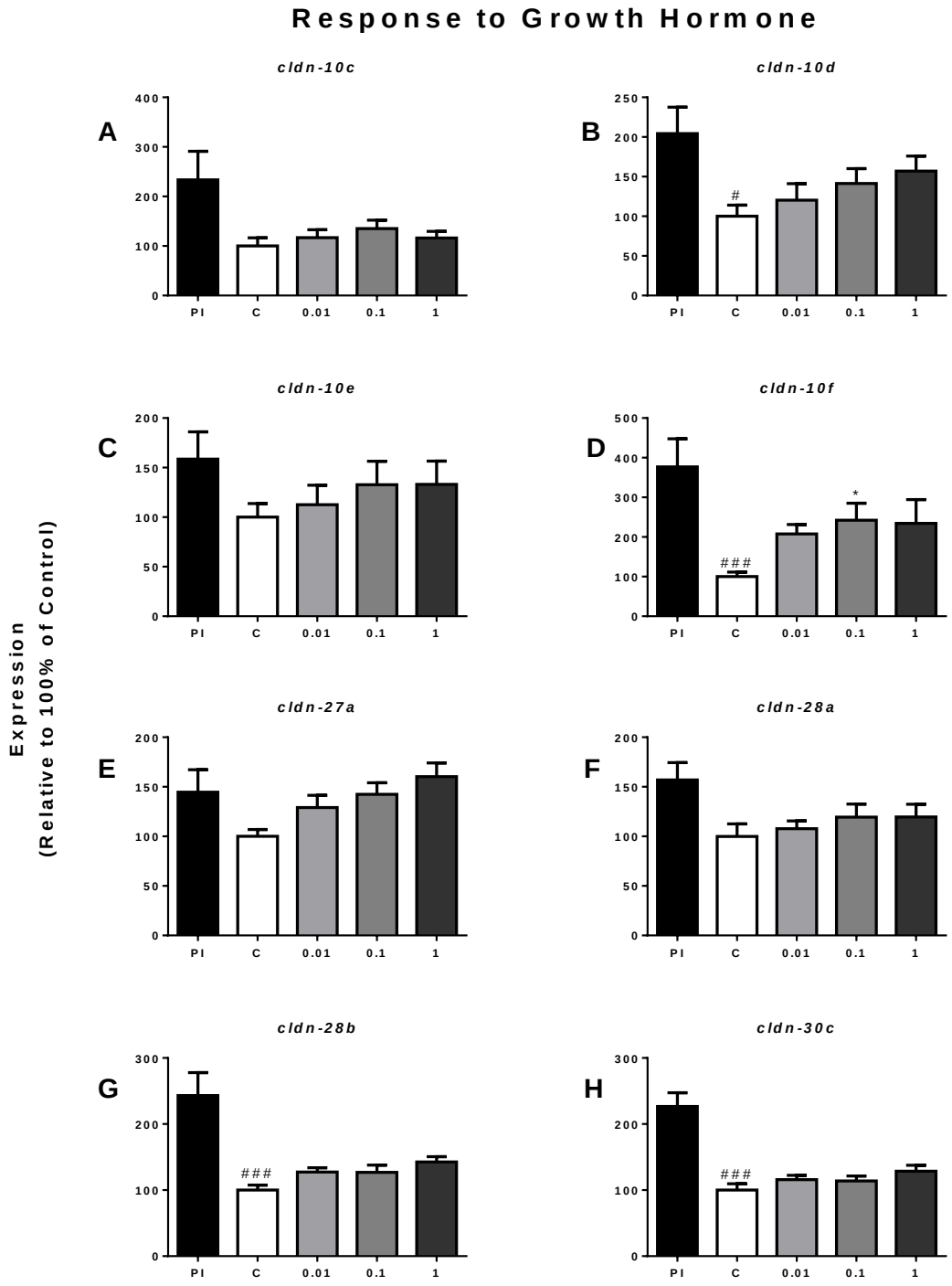
Response to Growth Hormone
Expression
(Relative to 100% of Control)
A
cldn-10c
400
300
200
100
0
PI
C
0.01
0.1
1
B
cldn-10d
250
200
150
100
50
0
#
C
cldn-10e
200
150
100
50
0
D
cldn-10f
500
400
300
200
100
0
###
*
E
cldn-27a
F
cldn-28a
G
cldn-28b
H
cldn-30c

Figure 8 (previous page). Response of *cldn-10c* (A), *-10d* (B), *-10e* (C), *-10f* (D), *-27a* (E), *-28a* (F), *-28b* (G), and *-30c* (H) expression to growth hormone. Results expressed with mean ± s.e.m. (N=6) relative to 100% of control expression. PI: preincubation control, C: control, numbers are indicative of the concentration of cortisol in μg/mL. Pound signs (#) are indicative of significance of the preincubation from the overnight incubation control, while asterisks (*) are indicative of significance of the concentration response from control. One symbol represents $p<0.05$ and three represents $p<0.001$.

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