Reverse Effect of Mammalian Hypocalcemic Cortisol in Fish: Cortisol Stimulates Ca\textsuperscript{2+} Uptake via Glucocorticoid Receptor-Mediated Vitamin D\textsubscript{3} Metabolism

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Abstract

Cortisol was reported to downregulate body-fluid Ca\textsuperscript{2+} levels in mammals but was proposed to show hypercalcemic effects in teleostean fish. Fish, unlike terrestrial vertebrates, obtain Ca\textsuperscript{2+} from the environment mainly via the gills and skin rather than by dietary means, and have to regulate the Ca\textsuperscript{2+} uptake functions to cope with fluctuating Ca\textsuperscript{2+} levels in aquatic environments. Cortisol was previously found to regulate Ca\textsuperscript{2+} uptake in fish; however, the molecular mechanism behind this is largely unclear. Zebrafish were used as a model to explore this issue. Acclimation to low-Ca\textsuperscript{2+} fresh water stimulated Ca\textsuperscript{2+} influx and expression of epithelial calcium channel (ecac), 11\textbeta-hydroxylase and the glucocorticoid receptor (gr). Exogenous cortisol increased Ca\textsuperscript{2+} influx and the expressions of ecac and hydroxysteroid 11-beta dehydrogenase 2 (hsd11b2), but downregulated 11\textbeta-hydroxylase and the gr with no effects on other Ca\textsuperscript{2+} transporters or the mineralocorticoid receptor (mr). Morpholino knockdown of the GR, but not the MR, was found to impair zebrafish Ca\textsuperscript{2+} uptake function by inhibiting the ecac expression. To further explore the regulatory mechanism of cortisol in Ca\textsuperscript{2+} uptake, the involvement of vitamin D\textsubscript{3} was analyzed. Cortisol stimulated expressions of vitamin D-25hydroxylase (cyp27a1), cyp27a1 like (cyp27a1l), 1x-Ohase (cyp27b1) at 3 dpf through GR, the first time to demonstrate the relationship between cortisol and vitamin D\textsubscript{3} in fish. In conclusion, cortisol stimulates ecac expression to enhance Ca\textsuperscript{2+} uptake functions, and this control pathway is suggested to be mediated by the GR. Lastly, cortisol also could mediate vitamin D\textsubscript{3} signaling to stimulate Ca\textsuperscript{2+} uptake in zebrafish.

Introduction

Corticosteroids (CSs) are primarily synthesized from cholesterol through a series of reactions. CSs, which consist of glucocorticoids (GCs) and mineralocorticoids (MCs), are vital hormones for mammals, and are involved in regulating the osmolality and ion levels, body fluids, energy metabolism, respiration, and immune reactions [1,2]. GCs are efficient treatment for asthma, rheumatoid arthritis, and atopic dermatitis because they can reduce immune responses; however, several systemic side-effects including osteoporosis are induced. An imbalance of Ca\textsuperscript{2+} handling is an important factor causing osteoporosis [3]. Regulation of Ca\textsuperscript{2+} absorption and emission is closely associated with the bone structure, and GC was reported to cause malabsorption and maldistribution of Ca\textsuperscript{2+} in the intestines and kidneys [2–4]. In mammals, GC was proposed to downregulate Ca\textsuperscript{2+} levels of body fluids through modulating the renal and duodenal expressions of TRPV6 and calbindin-D\textsubscript{9k} [5,6]. CS is synthesized in the adrenal cortex of mammals, but in the interrenal tissue of the head kidneys in teleosts. Physiological functions of CSs in teleosts are similar to those in mammals, and CS signaling is also mediated by the GC receptor (GR) and MC receptor (MR), which are ligand-activated transcription factors [7,8]. Both the GR and MR can bind the GC-responsive element (GRE) of the gene promoter and form GR-GR, MR-MR, and MR-GR dimers [9]. In CS synthesis, 11\textbeta-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2) are enzymes in the final step of the synthesis of cortisol and aldosterone, respectively. Teleosts may lack aldosterone synthase, and therefore cortisol is the main CS hormone in teleosts [10,11]. Some in vitro studies demonstrated that cortisol stimulated the transcriptional activity in mammalian cell lines transiently transfected with an expression construct containing a fish GR or MR and a reporter plasmid containing multiple GREs, implying that both teleostean CS receptors can be bound by cortisol with different affinities [12–15]. Based on these results, cortisol was suggested to have both GC and MC functions through different CS receptors, GR or MR, in teleosts; however, very few studies have investigated if the GR, MR, or both are involved in specific physiological processes in teleosts. In a recent study on Atlantic salmon, the GR and MR were found to differentially mediate the stimulation of various ion transporters in the gills during acclimation to salinity changes [16]. Vitamin D\textsubscript{3} was a vital calcitropic endocrine to regulate Ca\textsuperscript{2+} homeostasis in vertebrates. Liver vitamin D-25hydroxylase

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(CYP27A1) converts vitamin D₃ precursor to 25-hydroxyvitamin D₃ (25(OH)D₃), which is then converted to 1,25-dihydroxyvitamin D₃ (1,25 (OH)₂D₃), the active form of the vitamin D₃, by renal 1α-
OHase (CYP27B1) [17]. Vitamin D₃ spreads its function through its
receptor, vitamin D₃ receptor (VDR). VDR is a ligand-activated
transcription factor, and duodenal tpe6 (eca) is the target of the
vitamin D₃-VDR complex in mammals [10]. Stimulation of the
duodenal tpe6 expression by vitamin D₃ is one of important pathways
to enhance Ca²⁺ uptake in mammals [19]. In fish, vitamin D₃ had
been demonstrated to elevate serum Ca²⁺ levels [20,21]. Vitamin D₃
was also proposed to be associated with Ca²⁺ transport in the gills
based on the vitamin D₃ deficiency-induced impairment of growth
and mineralization in the fish skeleton [22,23]. Indeed, changes in the
plasma levels of 1,25 (OH)₂D₃ or mRNA expression of gill/kidney VDR
have been observed in salmon undergoing smolification and
migrating from freshwater (low Ca²⁺ concentrations) to seawater
(high Ca²⁺ concentrations), suggesting that synthesis of the sterol and
its receptor might be regulated depending upon ambient Ca²⁺
concentrations [23]. In mammals, glucocorticoid has been well
documented to affect vitamin D₃ metabolism although the actions
varied depending on species [24–29]. This suggests a possible
association between glucocorticoid, vitamin D₃, and Ca²⁺ homeostasis
in mammals; however, it is unknown whether this connection is also
developed in fish.

In teleosts, cortisol is well demonstrated to regulate the
mechanisms of ionic and osmotic balance, but only few studies
investigated the role of cortisol in controlling transepithelial Ca²⁺
transport [30,31]. Teleostean fish, unlike terrestrial vertebrates,
obtain Ca²⁺ mainly via absorption from the environment rather
than by dietary means. In adult fish, the predominant route of
Ca²⁺ entry from the environment is across the gill epithelium while
in larvae, the body skin is the major route of Ca²⁺ uptake before
full development of the gills occurs [32,33]. The Ca²⁺ uptake
function is well regulated for maintaining the internal Ca²⁺
homeostasis during acclimation to aquatic environments with
fluctuating Ca²⁺ levels (<0.01 mM in soft fresh water to >10 mM
in seawater) [30,34]. According to the current model in mammals
and teleosts, active tranacellular Ca²⁺ transport is carried out
through the operation of apical epithelial Ca²⁺ channels (ECaC,
TRPV5, and/or TRPV6), and basolateral plasma membrane
Ca²⁺-ATPase (PMCA) and the Na⁺/Ca²⁺ exchanger (NCX)
[34,35]. Exposure of rainbow trout to a reduced ambient calcium
level induces a rapid increase in systemic cortisol levels [36], and
exogenous cortisol can stimulate branchial ECaC mRNA and
protein expressions [37]. In an experiment with cultured gills,
cortisol was also found to enhance transepithelial Ca²⁺ transport
[38]. Those studies suggested a role of cortisol in control of Ca²⁺
uptake in teleosts; however, it is still unclear if cortisol controls only
ECaCs or other Ca²⁺ transporters (NCX and PMCA), and if this
control pathway is mediated by the GR, MR, or both. Elucidation
of these issues would enhance our understanding of the molecular
physiological mechanisms of cortisol’s control of epithelial Ca²⁺
transport, an essential component associated with bone structure
and formation in vertebrates.

Recently, zebrafish have become a model for research on ion
regulation and related endocrine controls due to the well-developed
genetic database and applicability of various molecular physiological
approaches [32,39]. In zebrafish gills and skin (in embryonic stages),
a specific ionocyte type that expresses ECaC, PMCA2, and
NCX1b was identified to be responsible for transepithelial Ca²⁺
uptake function [32,33,39–41], providing a suitable model to
further explore cortisol’s control of Ca²⁺ uptake mechanisms. The
present study attempted to address 3 specific questions: (I) Does
cortisol control zebrafish Ca²⁺ uptake function by regulating the
evca, ncx1b, and/or pmca2? (II) Does cortisol control zebrafish Ca²⁺
uptake function through mediation by the GR, MR, or both
receptors? (III) Does cortisol control zebrafish Ca²⁺ uptake function
by regulating vital calcitropic endocrine suchlike vitamin D₃? The
effects of environmental Ca²⁺ levels and exogenous cortisol on Ca²⁺
contents and influx, the mRNA expressions of Ca²⁺ transporters
(ecac, ncx1b, and pmca2), the steroidogenesis enzymes 11α-
hydroxysteroid dehydrogenase 2 (hsd11b2), CS receptors (gr and
mr) and vitamin D₃-related genes (vdra, vdh, cyp27a1, cyp27a1l
and cyp27b1) were investigated. Moreover, effects of knockdown of
the GR or MR on Ca²⁺ contents and influx, and the expression of
Ca²⁺ transporters and vitamin D₃-related genes in zebrafish
embryos were examined.

**Methods**

**Experiment animals**

Zebrafish (*Danio rerio*) were kept in local tap water
([Ca²⁺]= 0.2 mM) at 28.5 °C under a 14:10-h light-dark photoperiod
at the Institute of Cellular and Organismic Biology, Academia
Sinica, Taipei, Taiwan. Experimental protocols were approved by
the Academia Sinica Institutional Animal Care and Utilization
Committee (approval no.: RFIZOOHP220782).

**Acclimation experiments**

Artificial fresh waters with high- (2 mM) and low-Ca²⁺
(0.02 mM) levels were prepared with double-deionized water
(model Milli-RO60; Millipore, Billerica, MA, USA) supplemented
with adequate CaSO₄•2H₂O, MgSO₄•7H₂O, NaCl, K₂HPO₄,
and KH₂PO₄. Ca²⁺ concentrations (total Ca²⁺ levels measured by
absorption spectrophotometry) of the high- and low-Ca²⁺ media
were 2 and 0.02 mM, respectively, but the other ion concentra-
tions of the 3 media were the same ([Na⁺], 0.5 mM; [Mg²⁺],
0.16 mM; and [K⁺], 0.3 mM) as those in local tap water.
Variations in ion concentrations were maintained within 10% of
the predicted values. Fertilized zebrafish eggs were transferred to
high- and low-Ca²⁺ media, respectively, and incubated thereafter
until sampling at 3 d post-fertilization (dpf). The sampling time
in this study was based previously [42].

**Cortisol incubation experiments**

For cortisol incubation experiments, we based cortisol dosage
from previous study [43]. Cortisol (hydrocortisone, H8881, Sigma
Chemical Co., St Louis, MO, USA) was dissolved in local tap
water at 0 [control], 20, and 40 mg/L. Zebrafish embryos were
incubated in the cortisol media immediately after fertilization, and
were sampled at 1 or 3 dpf for the subsequent analysis. The
incubation media were changed with new cortisol solution every
day to maintain constant levels of cortisol. During incubation,
neither significant mortality nor abnormal behavior was found.

**Whole-body Ca²⁺ content**

Zebrafish embryos were anesthetized with MS-222 (Sigma) and
then briefly rinsed in deionized water. 30 individuals were pooled
as 1 sample. HNO₃ (13.1 N) was added to samples for digestion at
60°C overnight. Digested solutions were diluted with double-
deionized water, and the total Ca²⁺ content was measured with a
Z-8000 atomic absorption spectrophotometer (Hitachi, Tokyo,
Japan). Standard solutions (Merck, Darmstadt, Germany) were
used to make the standard curves.

**Whole-body Ca²⁺ influx**

By following previously described methods [44] with some
modifications, zebrafish embryos were dechorionated, rinsed
briefly in deionized water, and then transferred to 2 ml of $^{15}$Ca$^{2+}$ (Amersham, Piscataway, NJ; with a final working specific activity of 1–2 mCi/mmol)-containing medium for a subsequent 4-h incubation. After incubation, embryos were washed several times in isotope-free water medium. Six embryos were pooled into 1 vial, anesthetized with MS-222, and digested with tissue solubilizer (Solvable; Packard, Meriden, CT, USA) at 60°C for 0 h. The digested solutions were supplemented with counting solution ([Ultima Gold; Packard], and the radioactivities of the solutions were counted with a liquid scintillation beta counter (LS6500; Beckman, Fullerton, CA, USA). The Ca$^{2+}$ influx was calculated using the following formula:

$$J_m = \frac{Q_{embryo} X_{out} - 1}{t - 1} W^{-1},$$

where $J_m$ is the influx (pmol·mg$^{-1}$·h$^{-1}$), $Q_{embryo}$ is the radioactivity of the embryo (cpm per individual) at the end of incubation, $X_{out}$ is the specific activity of the incubation medium (cpm/pmol), $t$ is the incubation time (h), and $W$ is the average body wet weight of different-stage embryos (mg).

**RNA extraction**

After anesthetized with 0.03% MS222, appropriate amounts of zebrafish tissues or embryos were collected and homogenized in 1 ml Trizol reagent (Invitrogen, Carlsbad, CA, USA), then mixed with 0.2 ml chloroform and thoroughly shaken. After centrifugation at 4°C and 12,000g for 30 min, the supernatants were obtained. The samples were then mixed with an equal volume of isopropanol. Pellets were precipitated by centrifugation at 4°C and 12,000g for 30 min, washed with 70% alcohol, and stored at −20°C until use.

**Reverse-transcription polymerase chain reaction (RT-PCR) analysis**

For complementary (c)DNA synthesis, 1–5 μg of total RNA was reverse-transcribed in a final volume 20 μl containing 0.5 mM dNTPs, 2.5 μM oligo (dT)20, 250 ng random primers, 5 mM dithiothreitol, 40 units RNase inhibitor, and 200 units Superscript RT (Invitrogen) for 1 h at 50°C followed a 70°C incubation for 15 min. For PCR amplification, 2 μl cDNA was used as template in a 50-μL final reaction volume containing 0.25 mM dNTPs, 2.5 units Taq polymerase (Takara, Shiga, Japan), and 0.2 μM of each primer (Table S1). 30 cycles were performed for each reaction. All amplicons were sequenced to ensure that the PCR products were the desired gene fragments.

**Quantitative real-time PCR (qPCR)**

qPCR was performed with a LightCycler real-time PCR system (Roche, Penzberg, Germany) in a final volume of 10 μl, containing 5 μl 2× SYBR Green I Master (Roche Applied System), 300 nM of the primer pairs, and 20–30 ng cDNA. The standard curve for each gene was checked in a linear range with β-actin as an internal control. The primer sets for the qPCR are shown in Table S2.

**In situ hybridization**

Zebrafish ecac or gr Fragments were obtained by PCR and inserted into the pGEM-T easy vector (Promega, Madison, WI, USA). The inserted fragments were amplified with the T7 and SP6 primers by PCR, and the products as templates were used for the in vitro transcription with T7 and SP6 RNA polymerase (Roche) in the presence of digoxigenin (DIG)-UTP (Roche) to, respectively, synthesize sense and anti-sense probes. Zebrafish embryos were anesthetized on ice and fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; 1.4 mM NaCl, 0.2 mM KCl, 0.1 mM Na$_2$HPO$_4$, and 0.002 mM KH$_2$PO$_4$; pH 7.4) solution at 4°C overnight. To do in situ hybridization, we followed previously [40]. For the quantification of density, eight areas (85×80 μm$^2$ each) on the yolk sac surface of an embryo were chosen for counting.

**Organ culture**

Adult fish were anesthetized with 0.03% MS222 and then gills were dissected and directly transferred to the pre-incubation DMEM medium (Invitro) containing 50 mg/ml of penicillin (Invitro), 50 μg/ml of streptomycin (Invitro) and 20% Fetal Bovine Serum (Invitro). Individual gill arches were carefully separated from the whole gill structure. Each gill arch was cut lengthwise, and the cut filament was designated as one sample to be incubated in a well (96-well). The cut gill filaments were incubated with the freshly prepared pre-incubation medium (control group) and the DMEM with supplementary 20 mg/l cortisol, respectively. The media were freshly prepared and replaced twice per day. Organ culture was carried out at 28°C for 1 d in 96-well culture plates in a humidified chamber supplied with 95% O$_2$ and 5% CO$_2$.

**Morpholino oligonucleotide (MO) knockdown and rescue**

The zebrafish MR MO (5′-GTATCTTTTGTAGCTTCCGT-3′) and GR MO (5′-TCCAGTCTTCCTGTGATCCGT-3′) were prepared with 1× Danieae solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO$_4$, 0.6 mM Ca(NO$_3$)$_2$, and 5.0 mM HEPES; pH 7.6). A standard control MO (5′-CTCTTACCTCAGTTA-CATTTTATA-3′) was used as the control. To confirm MO specificity, fragments of the GR and MR containing the MO-targeted sequences were PCR-amplified with gene-specific primers (Table S3) and then cloned into the pCS2+GFP XLT vector, and the expression constructs were linearized to synthesize capped mRNA (cRNA) using an SP6 message RNA polymerase kit (Ambion, Austin, TX, USA). To confirm safety and efficiency of MOs and cRNA, we tested to inject various dosages of MOs and cRNA. Finally, we choose 2 ng/embryo (for MO) and 300 pg/embryo (for cRNA) to inject. Under these dosages, neither significant mortality nor abnormal behavior was found. The MO (2 ng/embryo) and/or cRNA (300 pg/embryo) were injected into embryos at the 1–2 cell stage using an IM-300 microinjection system (Narishige Scientific Instrument Laboratory, Tokyo, Japan). Green fluorescent protein (GFP) signals in 1-dpf embryos were observed by fluorescence microscopy (Axioplan 2 Imaging; Carl Zeiss, Oberkochen, Germany). MO-injected embryos at 1 or 3 dpf were sampled for subsequent analyses.

To rescue the defects caused by the MO, a full-length GR was PCR-amplified with a specific primer (Table S3) and cloned into the pCS2+ vector, and the construct was used to synthesize cRNA. Another GR MO, GR-SB MO, was designed at an intron-exon boundary by following a previous study [45], and this MO could only block endogenous GR translation. The full-length GR cRNA(300 pg/embryo) and GR-SB MO (5′-CGCTTTAGT-TATTTAGG-3′; 2 ng/embryo) were injected into embryos at the 1–2 cell stage, and embryos were sampled at 3 dpf.

**Western blot analysis**

Thirty embryos were pooled as one sample and homogenized. Protein of 50 mg/well was loaded to a 10% SDS-PAGE at 100 V for 2 h. After separation, proteins were transferred onto polyvinylidene difluoride membrane (Millipore) at 100 V for 2 h. After being blocked for 1.5 h in 5% nonfat milk, the blots were incubated with GR (Santa Cruz Biotechnology) or MR polyclonal antibody (Abcam) overnight 4°C, diluted 1:500 and with an alkaline-phos-phatase-conjugated goat anti-rabbit IgG (diluted 1:2500, room temp-perature; Jackson Laboratories) for
another 2 h. The blots were developed with 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium.

Cryosectioning

Fresh zebrafish gills were fixed with 4% PFA at 4°C for 3 h and then immersed serially in PBS containing 3, 10, and 20% sucrose for 15 min at room temperature. Finally, gills were soaked in a mixed PBS solution (OC:T compound: 20% sucrose at 1:2) overnight and then embedded with OCT compound embedding medium (Sakura, Tokyo, Japan) at 20°C. Cryosections at 6 μm were made with a cryostat (CM 1900; Leica, Heidelberg, Germany), and these were placed onto poly-L-lysine-coated slides (EMS, Hatfield, PA).

Immunocytochemistry

Prepared slides were rinsed in PBS and blocked with 3% BSA for 30 min. Afterward, the slides were first incubated with an α5 monoclonal antibody against the a-subunit of the avian Na,K-ATPase (NKA) (Hybridoma Bank, University of Iowa, Ames, IA; 1:600 dilution) overnight at 4°C. The slides were washed twice with PBS and incubated with an Alexa Fluor 568 goat anti-mouse IgG antibody (Molecular Probes, Carlsbad, CA; 1:200 diluted with PBS) for 2 h at room temperature. Images were acquired with a Leica TCS-NT confocal laser scanning microscope (Leica) or an Axiosplan 2 imaging microscope.

Potential regulatory elements upstream of the zebrafish ecac gene

The zebrafish ecac genomic sequence was obtained from a zebrafish genome database (http://www.ncbi.nlm.nih.gov/projectsgenome/guide/zebrafish/). Potential regulatory elements upstream of the ecac gene were predicted by Genomatix MatInspector (http://www.genomatix.de).

Statistical analysis

Data are presented as the mean±SD and were analyzed by one-way ANOVA and Student’s t-test.

Results

mRNA expressions of mr and gr

Both mr and gr mRNAs were universally expressed in all tissues studied (Fig. 1A). In developing embryos, mRNA expression of the gr was first detected at 1 h post-fertilization (hpf) and throughout development; however, mr mRNA only began to be expressed at 12 hpf (Fig. 1B).

Ca²⁺ incubation on Ca²⁺ influx and Ca²⁺-related genes

After acclimation to artificial fresh water containing different levels of Ca²⁺ for 3 d, zebrafish Ca²⁺ influx was significantly stimulated by low-Ca²⁺ water (Fig. 2A). Similarly, ecac, 11β-hydroxylase and gr mRNA expressions were also significantly stimulated by low-Ca²⁺ water (Fig. 2B). On the contrary, pmca2, nxc1b, hsd11b2, and mr mRNA expressions were not affected by environmental Ca²⁺ levels (Fig. 2B).

Exogenous cortisol on Ca²⁺ influx/contents and mRNA expressions of Ca²⁺-related target genes

To test the hypothesis of whether cortisol can affect Ca²⁺ uptake, zebrafish embryos at the 1–2-cell stage were treated with exogenous cortisol for 3 d. Incubation with exogenous cortisol caused dose-dependent effects on both Ca²⁺ contents and influx in 3-dpf zebrafish embryos. Compared to the control group (0 mg/l), cortisol-treated groups (20 and 40 mg/l) showed significant increases in Ca²⁺ content and influx (Fig. 3A, B). The qPCR revealed differential effects of exogenous cortisol on the mRNA expressions of Ca²⁺ transporters. mRNA expressions of zebrafish nxc1b and pmca2 were not affected by cortisol treatment (Fig. 3C); however, that of ecac was significantly upregulated by exogenous cortisol in a dose-dependent pattern (Fig. 3C). Furthermore, exogenous cortisol was also used to treat cultured gills. Similarly, ecac mRNA expression was affected by exogenous cortisol in gills, but nxc1b and pmca2 were not affected (Fig. S1). To support the data (Fig. 3C) of qPCR analyses for Ca²⁺ transporter expressions, in situ hybridization of ecac was conducted in the embryos treated with cortisol. As shown in Fig. 4A and B, exogenous cortisol significantly stimulated the density of ecac-expressing cells in 3-dpf zebrafish embryos. Exogenous cortisol also caused differential effects on mRNA expressions of 11β-hydroxylase, hsd11b2, gr, and mr in zebrafish embryos. According to the qPCR in 3-dpf embryos, exogenous cortisol significantly inhibited mRNA expressions of 11β-hydroxylase (in a dose-dependent manner) and gr and stimulated hsd11b2, but did not affect that of the mr (Fig. 3C).

Figure 1. Zebrafish mr and gr expression profiles. Determined by RT-PCR, mr and gr mRNA in various tissues of adults (A), and during developmental stages of embryos (B). β-actin was used as the internal control. doi:10.1371/journal.pone.0023689.g001

Loss-of-function effects on Ca²⁺ contents/influx/transporters and density of ecac-expressing cells in zebrafish embryos

To block the endogenous cortisol signaling pathway, MR and GR MOs were used to respectively inhibit translation of zebrafish GR and MR. The specificity and effectiveness of the MR and GR MOs were respectively confirmed by co-injection with zebrafish MR or GR cRNAs. Zebrafish embryos injected with only cRNAs (with GFP fusion) revealed signals of GFP translation (Fig. 5A, B), confirming the translation of MR and GR cRNAs. On the other hand, embryos co-injected with the MR (or GR) MO and MR (or GR) cRNA with GFP showed no green fluorescence (Fig. 5C, D), indicating that the MO specifically and effectively knocked-down the translation of MR (or GR) mRNA. In addition, Western blot was also used to further demonstrate MO specificity. As a result, GR or MR MO was found to specifically downregulate GR or MR protein level in 3-dpf zebrafish embryos (Fig. 5E).

After specificity tests, respective MOs were injected into 1–2-cell embryos. Compared to the control MO, the GR MO caused
Figure 2. Ca²⁺ influx and gene expressions of Ca²⁺ regulation-related genes. Ca²⁺ influx (A) and mRNA expression (B) of 3-dpf zebrafish embryos acclimated to low- (0.02 mM Ca²⁺) or high-Ca²⁺ (2.00 mM Ca²⁺) artificial fresh water. mRNA expression analyzed by qPCR and values were normalized to β-actin. Values are the mean ± SEM (n=4–6). *Significant difference (Student’s t-test, p<0.05).

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Figure 3. Effects of exogenous cortisol in 3-dpf zebrafish embryos. Ca²⁺ content (A), Ca²⁺ influx (B) and mRNA expressions (C). mRNA expressions were analyzed by qPCR, and values were normalized to β-actin. *Indicate a significant difference (p<0.05) using Tukey’s multiple-comparison test following one-way ANOVA. Value are the mean ± SEM (n=6 or 7).

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significant increases in Ca\textsuperscript{2+} content and influx in 3-dpf zebrafish embryos, but the MR MO had no effects (Fig. 6A, B). The qPCR assay of the mRNA expressions of Ca\textsuperscript{2+} transporters showed that the GR MO significantly reduced expression of the ecac, but did not affect ncx1b and pmca2 mRNA expressions in 3-dpf zebrafish embryos (Fig. 6C).

To further support these data, mRNA density of ecac-expressing cells in the skin of zebrafish morphants were analysed. The ecac-expressing cell was also significantly decreased upon GR MO injection (Fig. 7A, B). In contrast with the GR MO, the MR MO did not affect the expressions of ncx1b, pmca2, and ecac genes as well as the density of ecac-expressing cells in 3-dpf zebrafish embryos (Fig. 6C, 7A, B).

Effects of MR/GR MO on Ca\textsuperscript{2+} influx and ecac mRNA expression in zebrafish embryos with exogenous cortisol or low Ca\textsuperscript{2+} media

To precisely ascertain the different roles of the zebrafish GR and MR, the zebrafish were incubated with or without cortisol (20 mg/l) after injections with the MOs. Compared to the control group (control MO injection without cortisol), both groups of the control MO with cortisol and the MR MO with cortisol exhibited a significantly higher Ca\textsuperscript{2+} influx at 3-dpf, but the GR MO-injected embryos with exogenous cortisol did not (Fig. 8A). Similarly, ecac mRNA expression in the control MO with cortisol and MR MO with cortisol was significantly stimulated, while that of the GR MO-injected embryos was not affected by exogenous cortisol (Fig. 8B).

To further support the role of the GR in the Ca\textsuperscript{2+} uptake mechanism, the zebrafish GR-SB MO and/or GR cRNA were co-injected into zebrafish 1~2-cell-stage embryos. Compared to the control MO-injected group, GR-SB MO, similar to GR MO, also caused significant decreases in both Ca\textsuperscript{2+} influx and ecac mRNA expression in 3-dpf zebrafish embryos (Fig. 9A, B); however, co-injection with zebrafish GR cRNA and the GR-SB MO rescued the defective Ca\textsuperscript{2+} influx and ecac mRNA expression caused by the injection of the GR-SB MO (Fig. 9A, B).

Low Ca\textsuperscript{2+} medium was known to stimulate ecac expression in zebrafish [40,41] (Fig. 10). Whether this ecac expression upregulation by low Ca\textsuperscript{2+} medium is mediated by GR or MR was further clarified in the following experiments. One~two-cell-stage embryos were injected with the control MO, MR MO, and GR MO, respectively, and then were incubated in 2.0 mM (high) or 0.02 mM (low) Ca\textsuperscript{2+} medium. Compared to the control MO in low Ca\textsuperscript{2+} medium, the GR morphants in low Ca\textsuperscript{2+} medium were significantly lower in the ECaC mRNA expression at 3 dpf, but the MR morphants in low Ca\textsuperscript{2+} medium were similar to the control group (Fig. 10).

Potential regulatory elements in the zebrafish ecac gene

The 5’ flanking region of the zebrafish ecac gene was putatively identified and analyzed to search for possible regulatory elements. Several hormone-responsive elements, including the GRE, were identified within the 1653 bp analyzed in the putative upstream promoter region of the ecac gene (Fig. S2).

Exogenous cortisol on mRNA expressions of ecac and vitamin D\textsubscript{3}-related genes in zebrafish embryos

To investigate the effect of cortisol on the vitamin D\textsubscript{3}-related genes, exogenous cortisol (20 mg/l) was used to treat zebrafish
embryos. To trace the effect of exogenous cortisol, 1- and 3-dpf embryos were sampled. Cortisol caused differential effects on mRNA expressions of $\text{ecac}$ and the vitamin D3-related genes in 1- and 3-dpf zebrafish embryos. According to the qPCR, cortisol significantly stimulated $\text{ecac}$ and $\text{cyp27b1}$ mRNA expression in 1- and 3-dpf zebrafish embryos; however, cortisol only significantly stimulated the mRNA expressions of vitamin D3 receptor a ($\text{vdra}$), $\text{cyp27a1l}$ and $\text{cyp27a1}$ in 1- or 3-dpf embryos (Fig. 11A, B). On the other hand, $\text{vdrb}$ was not affected by cortisol in 1- and 3-dpf zebrafish embryos (Fig. 11A, B).

Effects of MR/GR MOs on mRNA expressions of the vitamin D3-related genes in zebrafish embryos

To further support the data of Fig. 7, 1-2-cell-stage embryos were injected with the control MO, MR MO, and GR MO, respectively. MR MO did not cause any effects on mRNA expressions of the vitamin D3-related genes in 1- and 3-dpf zebrafish embryos (Fig. 12A, B). On the contrary, GR MO caused differential effects on those vitamin D3-related genes (Fig. 12A, B). Only $\text{cyp27b1}$ mRNA expression was downregulated by GR MO at 1 dpf (Fig. 12A), but the mRNA expressions of $\text{vdra}$, $\text{cyp27a1l}$ and $\text{cyp27a1}$ were all decreased by GR MO at 3 dpf (Fig. 12B).

Discussion

Glucocorticoid (cortisol) showed different impact in Ca$^{2+}$ handling between mammal and fish; however, the understanding is little of cortisol control in fish. For this purpose, we explored cortisol effect in zebrafish Ca$^{2+}$ handling. In the present study, the major findings were the following: (I) expression of zebrafish 11$\beta$-hydroxylase was stimulated by low-Ca$^{2+}$ environment; (II) exogenous cortisol increased zebrafish Ca$^{2+}$ influx and content through upregulating the expression of $\text{ecac}$ but not those of $\text{ncx1b}$ or $\text{pmca2}$; (III) exogenous cortisol probably through a feedback pathway modulated the mRNA expression of 11$\beta$-hydroxylase, $\text{hsd11b2}$ and $\text{gr}$ but not that of $\text{mr}$ in zebrafish; and (IV) translational knockdown of...
GR but not MR caused defects in the binding of cortisol or mRNA expression of vitamin D3-related genes, and thus impaired the expression and Ca\(^{2+}\) uptake function of zebrafish ECaC via the GR, a regulatory pathway that could be mediated by vitamin D3.

Fish mainly obtain Ca\(^{2+}\) from the aquatic environment with fluctuating Ca\(^{2+}\) levels, and therefore the internal Ca\(^{2+}\) homeostasis is impacted by environmental Ca\(^{2+}\) levels. Fish (at least teleost) bone is acellular, and thus the bone does not provide a pool for Ca\(^{2+}\) as it does in terrestrial vertebrates [30]. For internal Ca\(^{2+}\) homeostasis and bone formation (particularly in embryonic and larval stages), fish have to regulate the function of Ca\(^{2+}\) uptake to cope with a fluctuating environment. Like other teleosts, zebrafish can enhance Ca\(^{2+}\) uptake function by stimulating ECaC expression during acclimation to low-Ca\(^{2+}\) fresh water [40,41,44], and this functional regulation may be associated with cortisol. Flik and Perry [36] reported that acclimation to low-Ca\(^{2+}\) fresh water caused an increase in serum cortisol levels in rainbow trout. The present study further explored the mechanism behind this phenomenon. Low-Ca\(^{2+}\) fresh water stimulated the mRNA expression of 11\(\beta\)-hydroxylase, the enzyme in the final step of cortisol synthesis, which suggests that environmental conditions affect steroidogenesis and thus cortisol levels in zebrafish. All these results imply a possible role of cortisol in the control of Ca\(^{2+}\) uptake. To test this hypothesis, we treated zebrafish with exogenous cortisol. Similar to trout and eel [37,46], in zebrafish, exogenous cortisol stimulated the mRNA expression of ecac and Ca\(^{2+}\) influx, and these functional enhancements resulted in increased Ca\(^{2+}\) contents in the whole body (the present study).

The present comprehensive data from molecular to the physiological level demonstrated the calciotropic effects of cortisol, and these effects showed a dose-dependent pattern and were of physiological significance.

The ECaC, NCX1b, and PMCA2 are coexpressed in a specific type of ionocyte [41], which achieves the epithelial Ca\(^{2+}\) uptake function through the operations of the 3 transporters in zebrafish [32,33,39]. Previously, exogenous cortisol was found to stimulate branchial ECaC mRNA and protein expressions in trout [37], but

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**Figure 8.** Effects of MR MO and GR MO on zebrafish embryos with cortisol treatment. Ca\(^{2+}\) influx (A) and ecac mRNA expression (B) were analyzed in 3-dpf zebrafish embryos injected with GR MO or MR MO with cortisol treatment. mRNA expressions were analyzed by qPCR, and values were normalized to \(\beta\)-actin. abcIndicate a significant difference (\(p<0.05\)) using Tukey’s multiple-comparison test following one-way ANOVA. Values are the mean ± SEM. (\(n=6–8\)).

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**Figure 9.** Effects of GR cRNA on GR-SB MO-injected zebrafish embryos. Ca\(^{2+}\) influx (A) and ecac mRNA expression (B) were also analyzed in 3-dpf zebrafish embryos injected with GR-SB MO or GR-SB MO with GR cRNA. mRNA expressions were analyzed by qPCR, and values were normalized to \(\beta\)-actin. abcIndicate a significant difference (\(p<0.05\)) using Tukey’s multiple-comparison test following one-way ANOVA. Values are the mean ± SEM. (\(n=6–8\)).

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no attempt was made to examine the effects on the other Ca\(^{2+}\) transporters (NCX and PMCA). The present study first reports that exogenous cortisol affected eac\(^+\) only but not those of ncc1b or pmca2; similar result found in cultured gills with exogenous cortisol (Fig. S1). On the other hand, we further directly demonstrated eac-expressing cell density in skin of zebrafish embryos was stimulated by exogenous cortisol. These results supported the previous notion that the ECaC is the rate-limiting step and the gatekeeper channel for active Ca\(^{2+}\) transport in fish [39] as in mammals [47]. On the other hand, translational knockdown of stanniocalcin, a hypocalcemic hormone, was reported to stimulate eac expression and Ca\(^{2+}\) influx, but not affect the expressions of ncc1b or pmca2 in zebrafish [42]. Similarly in the present study, knockdown of the GR downregulated the expression of eac, but showed no effect on ncc1b or pmca2. The eac appears to be the major regulatory target gene in response to environmental Ca\(^{2+}\) levels and also the upstream control of hormones in zebrafish.

In the present study, exogenous cortisol suppressed the mRNA expression of 11\(\beta\)-hydroxylase (decreasing the cortisol level) and simultaneously stimulated that of hsd11b2 (increasing the cortisone level), reflecting a feedback mechanism in controlling levels of corticoids. In mammals, HSD11B2 converts cortisol to cortisone in MR-specific tissues, and this prevents cortisol binding to the MR in these tissues and thus allows aldosterone to bind to the MR [48]. In fish, HSD11B2 can also convert cortisol to cortisone [49,50]. Thus, stimulation of hsd11b2 by exogenous cortisol treatment may be a feedback to control cortisol levels in zebrafish. This notion was further supported by the data of 11\(\beta\)-hydroxylase and the gr; gene expressions of zebrafish 11\(\beta\)-hydroxylase and the gr were inhibited by exogenous cortisol treatment. In mammals, dexamethasone treatment suppressed the secretion of the adrenocorticotrophic hormone, resulting in a decline in serum corticosterone levels [51,52], and the duodenal and renal gr expressions were significantly downregulated by dexamethasone treatment [6]. Similarly in trout and salmon, cortisol treatment by infusion, feeding, or soaking also caused downregulation of CS receptors or the gr in gills and liver [53–57]. Taken together, cortisol may regulate the function of the Ca\(^{2+}\) mechanism through sophisticated feedback pathways, in which the expressions of 11\(\beta\)-hydroxylase, hsd11b2, and CRs are differentially modulated.

Physiological functions of cortisol signaling are mediated by the GR and MR, which are ligand-activated transcription factors. In many previous studies, exogenous cortisol treatment was found to stimulate Ca\(^{2+}\) uptake in teleosts [36,38,46]; however, it was unknown until the present study that cortisol controls the Ca\(^{2+}\) uptake function through the GR but not the MR. In addition to experiments of exogenous cortisol treatment, we used a gene-specific MO to abolish endogenous cortisol signaling in zebrafish and directly explored the effect of cortisol-signaling defects on the Ca\(^{2+}\) uptake function. Translational knockdown of the MR did not affect Ca\(^{2+}\) uptake in zebrafish, but GR knockdown evidently impaired the Ca\(^{2+}\) uptake mechanism by decreasing both Ca\(^{2+}\) influx and content. Moreover, these defects in the Ca\(^{2+}\) uptake mechanism were due to suppression of eac expression but not the expressions of ncc1b or pmca2. GR defect was also demonstrated to downregulate eac-expressing cell density in skin of zebrafish. These results indicated that the target of the GR is the eac. Interestingly, the Ca\(^{2+}\) influx, eac mRNA and eac-expressing cell density in MR MO-injected zebrafish morphants could still be stimulated (compared to the control) by exogenous cortisol, suggesting that the effects of exogenous cortisol on Ca\(^{2+}\) uptake function is not through the MR. On the other hand, exogenous cortisol did not cause further stimulation (compared to the control) in Ca\(^{2+}\) influx or eac mRNA in GR morphants, but successfully rescued the Ca\(^{2+}\) uptake functional defects caused by the GR MO. Similar effect was also found in GR MO-injected zebrafish with low Ca\(^{2+}\) freshwater. GR MO morphant can abolish stimulation of low Ca\(^{2+}\) freshwater on eac mRNA expression, but MR MO morphant can not. Reinforcing these results, overexpression of the GR by injection with GR cDNA rescued the Ca\(^{2+}\) uptake mechanism that was impaired in GR MO morphants. This evidence strongly suggests that endogenous cortisol stimulates Ca\(^{2+}\) uptake through the GR, but not the MR, in zebrafish.

Some previous in vitro studies indicated that the trout MR and GR could bind cortisol and stimulate transcriptional activity in the mammalian cell lines transfected with a reporter plasmid [12–15]. In a recent study on Atlantic salmon, Kiilerich et al. [16] used GR and MR antagonists to discover that the 2 CS receptors were involved in regulating various ion transporters (NKA, NKCC, and CFTR) during acclimation to salinity changes. Differences between salmon (involvement of both the GR and MR) and zebrafish (only the GR) may be because of differences in the ion transport functions (Na\(^{+}\)/K\(^{+}\) vs. Ca\(^{2+}\)) and species (euryhaline vs. stenohaline), and clarification of this point requires further studies. On the other hand, it was noted that zebrafish MR expression was not regulated by exogenous cortisol in the present study. Teleosts might not synthesize aldosterone, and DOC was proposed to play a similar role as aldosterone [15]. Moreover, DOC was suggested to be a potent agonist of the fish MR because DOC can induce greater transcription activity than cortisol through the trout MR expressed in a mammalian cell line co-transfected with a reporter plasmid [15]. It will be challenging to see if a lack of an effect of exogenous cortisol on MR expression is due to different ligand affinities between the 2 CS receptors in zebrafish.

Cortisol shows hypercalcemic effects in zebrafish and other fish species as described above. On the contrary, cortisol was reported to inhibit the intestinal Ca\(^{2+}\) absorption in chickens [58], and GC drugs, dexamethasone and prednisolone, were demonstrated to inhibit duodenal Ca\(^{2+}\) uptake and top6 (eac) gene expression in

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**Figure 10.** Effect of MR MO and GR MO on eac mRNA expression with low Ca\(^{2+}\) treatment. eac mRNA expression was analyzed in 3-dpf zebrafish embryos injected with GR MO or MR MO with low Ca\(^{2+}\) (0.02 mM; LCa) treatment. mRNA expressions were analyzed by qPCR, and values were normalized to \(\beta\)-actin. *Indicate a significant difference (\(p=0.05\)) using Tukey’s multiple-comparison test following one-way ANOVA. Values are the mean ± SEM. (.6–8).

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mice [5,6]. Recently, Kim et al. [6] found only one GRE in the promoter region of mice trpv6, and suggested that dexamethasone might not directly regulate trpv6 transcription, but instead, downregulates the Ca\(^{2+}\) uptake function through other hormones. Based on a bioinformatics analysis, there are some putative GREs in the promoter region of the zebrafish ecac (Fig. S2). Previous in situ hybridization study on developing zebrafish embryos [59], gr transcript was detected over the skin where ionocytes appear. As shown in Fig. S3, GR was colocalized in NaR cells, which express ECaC [40]. These imply the possibility that cortisol can stimulate mRNA expression of the ecac through interaction with the GREs; however, further studies are needed to support this notion.

In addition to direct regulation of ecac transcript, cortisol may modulate the other endocrine to affect Ca\(^{2+}\) uptake in zebrafish. Vitamin D\(_3\) is a well-known calcitrophic endocrine to regulate Ca\(^{2+}\) homeostasis in vertebrates [19–21], and several mammal studies demonstrated that glucocorticoid could affect vitamin D\(_3\) metabolism [24–29]. Vitamin D\(_3\) can bind VDR, forming a vitamin D\(_3\)-VDR complex. This complex directly stimulates mammalian intestinal ecac transcript by binding vitamin D\(_3\) responsive element (VDRE) in the promoter region of ecac [18]. In fish, vitamin D\(_3\) was also reported to elevate the serum Ca\(^{2+}\) level [20,21], and putative VDRE was also identified in ecac promoter region [60] (Fig. S2). Recent in vitro study in medaka, VDR was found to simulate transcript level of VDRE-containing construct with 1,25(OH)\(_2\)D\(_3\) treatment [61]. Our unpublished data also indicated stimulation of ecac mRNA expression by 1,25(OH)\(_2\)D\(_3\) in zebrafish embryos. Taken together, vitamin D3-VDR control of ecac expression and function may also exist in fish. However, the associations between cortisol, vitamin D\(_3\) and Ca\(^{2+}\) handling in fish were not clear until the present study. CYP27A1 is an enzyme to synthesize 25(OH)D\(_3\), the vitamin D\(_3\) precursor, and subsequently 25(OH)D\(_3\) is converted to 1,25(OH)\(_2\)D\(_3\) (active vitamin D\(_3\)) by CYP27B1 [17]. In zebrafish embryos, exogenous cortisol could upregulate the expressions of cyp27b1, cyp27a1 and/or cyp27a1 like (cyp27a1l) at 1- and 3 dpf, and also affected vdra mRNA expression at 1 dpf. These implied that cortisol starts affecting the vitamin D\(_3\)-VDR signaling from early stage. Cortisol could not only stimulate synthesis of vitamin D\(_3\) precursors but also accelerate the synthesis of active vitamin D\(_3\). Moreover, our subsequent knockdown experiment further reinforced this notion. Knockdown of GR, but not MR, was found to suppress the expressions of cyp27b1, cyp27a1, cyp27a1l and vdra at 1- and/or 3 dpf. According to these results, we suggested that cortisol probably regulates Ca\(^{2+}\) handling through vitamin D\(_3\)-VDR system, besides directly regulating ecac expression.

In this study, cortisol was suggested to directly or indirectly stimulate Ca\(^{2+}\) uptake in zebrafish. Cortisol appears to cause different effects on Ca\(^{2+}\) uptake between teleosts and higher vertebrates. Sources of Ca\(^{2+}\) fundamentally differ among vertebrates. Sources of Ca\(^{2+}\) fundamentally differ among vertebrates.

**Figure 11. Effects of exogenous cortisol on mRNA expressions of the vitamin D\(_3\)-related genes.** qPCR was used to analyze mRNA expression and values were normalized to \(\beta\)-actin. (A) mRNA expressions in 1-dpf zebrafish embryos. (B) mRNA expressions in 3-dpf zebrafish embryos. *Indicate a significant difference (p≤0.05) using Tukey’s multiple-comparison test following one-way ANOVA. Values are the mean ± SEM (n = 6).

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For terrestrial vertebrates, food is the major source of Ca$^{2+}$, and the intestines serve as a primary site for Ca$^{2+}$ uptake. However, fish are constantly facing aquatic environments with variable Ca$^{2+}$ concentrations (of as low as <0.01 mM in soft fresh water), and gills or the skin serve as the primary site for Ca$^{2+}$ uptake [30,32,33]. These differences may be associated with different modes of cortisol control of Ca$^{2+}$ uptake, which is a challenging issue to be explored from an evolutionary point of view.

**Supporting Information**

**Table S1** Primers for the RT-PCR analysis. (DOC)

**Table S2** Primers for the qPCR analysis. (DOC)

**Table S3** Primers for cRNA expression cloning. (DOC)

**Figure S1** Effect of exogenous cortisol on mRNA expression of the vitamin D$_3$-related genes. (A) mRNA expressions in 1-dpf zebrafish embryos. (B) mRNA expressions in 3-dpf zebrafish embryos. mRNA expression was analyzed by qPCR and values were normalized to $\beta$-actin. *a*, *b* Indicate a significant difference ($p<0.05$) using Tukey’s multiple-comparison test following one-way ANOVA. Values are the mean ± SEM ($n=5$). (TIF)

**Figure S2** Upstream regulatory region of the zebrafish ecac gene. The transcription initiation sites are marked by +1, and the start codon (ATG) is marked by a square. The putative upstream regulatory elements are underlined. The core sequence of each element is shown in bold font. GRE, glucocorticoid-responsive element; VDRE, vitamin D$_3$-responsive element; ARE, androgen-responsive element. (TIF)

**Figure S3** Co-localization of gr mRNA by in situ hybridization with anti-NKA using immunocytochemical analysis of zebrafish gill cryosections. (A) in situ hybridization of gr mRNA; (B) immunocytochemical staining of NKA. Arrow indicated gr mRNA and NKA protein signals at similar area. Scale bar 5 μm. (TIF)

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**Author Contributions**

Conceived and designed the experiments: C-HL D-YT P-PH. Performed the experiments: C-HL I-LT C-HS. Analyzed the data: C-HL I-LT. Contributed reagents/materials/analysis tools: C-HL P-PH. Wrote the paper: C-HL P-PH.
References

1. Charmandari E, Tsigos C, Chrousos G (2005) Endocrinology of the stress response. Ann Rev Physiol 67: 259–284.
2. McLaughlin F, Mackintosh J, Hayes BP, McLaren A, Unger JJ, et al. (2002) Glucocorticoid-induced osteoporosis in the mouse as assessed by histomorphometry, microcomputed tomography, and biochemical markers. Bone 30: 924–930.
3. Patsch J, Loddenkemper K, Buttgerfeid F (2001) Molecular mechanisms of glucocorticoid-induced osteoporosis. Bone 29: 490–503.
4. Luiten PK, Raan LG (1990) Glucocorticoid-induced osteoporosis: pathogenesis and management. Ann Intern Med 112: 352–364.
5. Huybers S, Naber TH, Bindels RJ, Hoenderob GP (2007) Predisolone-induced Ca2+ malabsorption caused by diminished expression of the epithelial Ca2+ channel TRPV6. J Physiol Gastric Liver Physiol 29(2): 692–701.
6. Kim MH, Lee GS, Jung EM, Choi KC, Oh GT, et al. (2009) Dexamethasone differentially regulates renal and duodenal calcium-processing genes in cilia-deficient and -deficient knockout mice. Exp Physiol 94(13): 131–138.
7. Bury NR, Sturm A (2007) Evolution of the corticosteroid receptor signalling pathway in fishes. Gen Comp Endocrinol 153(1–3): 47–56.
8. Mølmen TP, Vijayan MM, Moon TW (1999) Cortisol in teleostic dynamics, mechanisms of action, and metabolic regulation. Rev Fish Biol Fish 9: 211–260.
9. Trapp T, Hebbeler F (1996) Heterodimerization between mineralocorticoid and glucocorticoid receptors increases the functional diversity of corticosteroid action. Trends Pharmacol Sci 17: 149–154.
10. Baker ME (2005) Evolution of Glucocorticoid and Mineralocorticoid Responses. In: Fish Endocrinology. 44: 4223–4237.
11. Nelson DR (2003) Comparison of P450s from human and fugu: 420 million years of vertebrate P450 evolution. Arch Biochem Biophys 409: 18–24.
12. Carman L, Fosco A, Bury N, Pekel F, Gourlet G, et al. (2000) A mineralocorticoid-like receptor in the rainbow trout, Oncorhynchus mykiss: cloning and characterization of its steroid binding domain. Steroids 65: 319–328.
13. Bury NR, Sturm A, Le Rouzic P, Lethimonier C, Ducouret B, et al. (2003) Evidence for two distinct functional glucocorticoid receptors in teleost fish. J Mol Endocrinol 31: 141–156.
14. Greenwood AK, Butler PC, White DR, DeMarco U, Pearce D, et al. (2003) Multiple corticosteroid receptors in a teleost fish: distinct sequences, expression patterns, and transrepression activities. Endocrinology 144: 4226–4236.
15. Sturm A, Bury NR, Dengreille L, Fagart J, Flennor G, et al. (2003) 11-Deoxycorticosterone is a potent agonist of the rainbow trout (Oncorhynchus mykiss) mineralocorticoid receptor. Endocrinology 144: 47–55.
16. Kährt P, Kristiansen K, Madsen SS (2007) Cortisol regulation of ion transporters in Atlantic salmon gill and the effect of salinity on the signalling pathway. J Endocrinol 194(2): 417–427.
17. DeLuca HF (1988) The vitamin D story: a collaborative effort of basic science and clinical medicine. J Bone Miner Res 3(4): 224–236.
18. Haasler MR, Whitfield GK, Haasler CA, Huijts JC, Thompson PD, et al. (1998) The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. Bone Miner Res 13: 325–349.
19. Dolhak MF (2007) Vitamin D Deficiency. N Engl J Med 357: 266–261.
20. Swarr SC, Dave VK, Norman AW (1991) Dose-dependent vitamin D3 and 1,25-(OH)2 vitamin D3-mediated inhibition of Ca2+ transport in the caecal malabsorption model induced by cyclophosphamide. J Endocrinol Liver Physiol 29(2): 692–701.
21. Abbink W, Hang XM, Guerreiro PM, Spanings FA, Ross HA, et al. (2007) Effect of dexamethasone treatment on maturational changes in the NMDA receptor mRNA in Atlantic salmon gill and the effect of salinity on the expression, cell morphology and calcium uptake in freshwater teleosts. Fish Physiol Biochem 33(3): R1490–R1498.
22. Lock EJ, Osmund K, Aknes I, Spanings FA, Waagbo R, et al. (2007) The vitamin D receptor and its ligand 1,25-dihydroxyvitamin D3 in Atlantic salmon (Salmo salar). J Endocrinol 193: 459–471.
23. Cherney RW, Mauers RB, Haasler AJ, DeLuca HF, O’Reagan S, et al. (1978) Reduction of serum-1,25-dihydroxyvitamin D3 in children receiving glucocorticoids. Lancet 312(1980): 1123–1125.
24. Seeman E, Kumar R, Hunder GG, Scott M, Heath H, 3rd, et al. (1980) Production, degradation, and circulating levels of 1,25-dihydroxyvitamin D3 in health and in chronic glucocorticoid excess. J Clin Invest 66(4): 664–669.
25. Morris HA, Need AG, O’Loughlin PD, Horowitz M, Bridges A, et al. (1990) Assimilation of calcium in corticosteroid-induced osteoporosis. Calcif Tissue Int 46(5): 305–308.
26. Prummel MF, Wiersinga WM, Lips P, Sanders GT, Sauerwein HP, et al. (1991) The course of biochemical parameters of bone turnover during treatment with corticosteroids. J Clin Endocrinol Metab 72(6): 456–461.
27. Fournier R, Nieweg OE, Ghezzi F, Neven J, Shnayder R, et al. (1994) High-dose glucocorticoids in multiple sclerosis patients exert direct effects on the kidney and skeleton. J Bone Miner Res 9(7): 1097–1105.
28. Evans DH, Piermarini PM, Choe KP (2005) The multifunctional fish gill: Domains of function, vertebrate fish. Proc Natl Acad Sci U S A 78(11): 3542–3546.
29. Shultz TD, Bollman S, Kumar R, 2002. Decreased intestinal calcium absorption in vivo and normal brush border membrane vesicle calcium uptake in cortisoldreated chickens: evidence for dissociation of calcium absorption from brush border membrane calcium transport. Proc Natl Acad Sci U S A 99(18): 10949–10953.
30. Shultz TD, Bollman S, Kumar R, 1982. Decreased intestinal calcium absorption in vivo and normal brush border membrane vesicle calcium uptake in cortisoldreated chickens: evidence for dissociation of calcium absorption from brush border membrane calcium transport. Proc Natl Acad Sci U S A 79(11): 3542–3546.
31. Shultz TD, Bollman S, Kumar R, 1982. Decreased intestinal calcium absorption in vivo and normal brush border membrane vesicle calcium uptake in cortisoldreated chickens: evidence for dissociation of calcium absorption from brush border membrane calcium transport. Proc Natl Acad Sci U S A 79(11): 3542–3546.
32. Shultz TD, Bollman S, Kumar R, 1982. Decreased intestinal calcium absorption in vivo and normal brush border membrane vesicle calcium uptake in cortisoldreated chickens: evidence for dissociation of calcium absorption from brush border membrane calcium transport. Proc Natl Acad Sci U S A 79(11): 3542–3546.
60. Qiu A, Hogstrand C (2004) Functional characterisation and genomic analysis of an epithelial calcium channel (ECaC) from pufferfish, *Fugu rubripes*. Gene 342(1): 113–123.

61. Howarth DL, Law SH, Barnes B, Hall JM, Hinton DE, et al. (2008) Paralogous vitamin D receptors in teleosts: transition of nuclear receptor function. Endocrinology 149(5): 2411–2422.

Cortisol Stimulates Ca\(^{2+}\) Uptake in Fish