Involvement of the calcium-sensing receptor in calcium homeostasis in larval zebrafish

exposed to low environmental calcium

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Running head: Role of the calcium-sensing receptor in zebrafish

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Abstract
The involvement of the calcium-sensing receptor (CaSR) in Ca\(^{2+}\) homeostasis was investigated in larval zebrafish, *Danio rerio*. The expression of CaSR mRNA was first observed at 3 h post-fertilization (hpf) and increased with development until plateauing at about 48 hpf. At 4 days post-fertilization (dpf), CaSR mRNA was increased in fish acclimated to low Ca\(^{2+}\) water (25 \(\mu\)M versus 250 \(\mu\)M in normal water). Using immunohistochemistry and confocal microscopy, it was demonstrated that the CaSR is expressed in the olfactory epithelium, neuromasts, ionocytes on the yolk sac epithelium, and corpuscles of Stannius. Results of double immunohistochemistry and/or *in situ* hybridization indicated that the CaSR is localized to a subset of mitochondrion-rich ionocytes enriched with Na\(^+/K^+\)-ATPase and epithelial Ca\(^{2+}\) channel (ecac). Translational knockdown of the CaSR prevented 4 dpf larvae from regulating whole-body Ca\(^{2+}\) levels when exposed to a low Ca\(^{2+}\) environment. Further, the increases in ecac mRNA expression and Ca\(^{2+}\) influx, normally associated with exposure to low Ca\(^{2+}\) water, were prevented by CaSR knockdown. These findings demonstrate that larval zebrafish lacking the CaSR lose their ability to regulate Ca\(^{2+}\) when confronted with a low Ca\(^{2+}\) environment. Results from real-time PCR suggested that the mRNA expression of the hypocalcemic hormone stanniocalcin (*stc-1*) remained elevated in the CaSR morphants following acclimation to low Ca\(^{2+}\) water. Overall, the results suggest that the CaSR is critical for Ca\(^{2+}\) homeostasis in larval zebrafish exposed to low environmental Ca\(^{2+}\) levels, possibly owing to its modulation of stanniocalcin mRNA expression.

Key words: calcium-sensing receptor; calcium homeostasis; ECaC; stanniocalcin; zebrafish
INTRODUCTION

Calcium is an essential element for growth and survival, and its levels are tightly regulated in vertebrates including fish. Unlike mammals which rely on diet as their main source of Ca\(^{2+}\), fish acquire the majority of their Ca\(^{2+}\) from the external environment and regulate internal Ca\(^{2+}\) levels by adjusting its absorption across the gill (adults) or integument (larvae). For example, fish acclimated to a low Ca\(^{2+}\) environment maintain whole-body Ca\(^{2+}\) balance by increasing their capacity to absorb Ca\(^{2+}\) from the water (7, 32, 35). The increase in Ca\(^{2+}\)-transporting capacity is associated with the proliferation of specific ion-transporting cells (ionocytes) on the gill (6, 35) or integument (32). In zebrafish (*Danio rerio*), Ca\(^{2+}\) uptake is accomplished by a subset of mitochondrion-rich ionocytes enriched with Na\(^+\)/K\(^-\)-ATPase (NaR cells), which express epithelial Ca\(^{2+}\) channels (ECaC) at the apical membrane, and plasma membrane Ca\(^{2+}\)-ATPase (PMCA) and Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) at the basolateral membrane (23, 32). The rate-limiting step in the uptake of Ca\(^{2+}\) across epithelia is generally considered to be its movement across the apical membrane via ECaC (34). In keeping with its pivotal role in Ca\(^{2+}\) uptake, *ecac* expression and the density of *ecac*-expressing cells were significantly increased in larval zebrafish acclimated to low Ca\(^{2+}\) water (32). However, the mechanism by which fish detect changes in the environmental Ca\(^{2+}\) availability and thereby modulate their Ca\(^{2+}\) transporting pathways is poorly understood. Previous studies have suggested that fish may respond to changes in environmental Ca\(^{2+}\) levels by activation/inactivation of the extracellular Ca\(^{2+}\)-sensing receptor (CaSR) (for review see 28).

The CaSR is a member of the G-protein-coupled seven trans-membrane-domain receptor superfamily (for review see 19); binding of Ca\(^{2+}\) to the extracellular domain of the CaSR elicits a downstream signalling cascade involving various protein kinases and phospholipases (3). It is well established that in mammals, where the CaSR is expressed in various tissues including the
kidney, intestine, bone and parathyroid gland (4, 19), that it is involved in regulating Ca\(^{2+}\) absorption and systemic Ca\(^{2+}\) handling (8). It was also demonstrated that mice fed a Ca\(^{2+}\)-enriched diet exhibited a reduction in Ca\(^{2+}\) uptake owing to reduced intestinal expression of Ca\(^{2+}\)-transport proteins (CaT), whereas CaT expression remained elevated in the CaSR-deficient mice fed the same diet (24). Furthermore, inactivation of the CaSR has been shown to reduce the capacity to increase urinary Ca\(^{2+}\) excretion in response to hypercalcemia (15).

In teleost fish, the CaSR is expressed in numerous tissues, including the gill, olfactory organ, kidney, intestine and corpuscles of Stannius (11, 16, 26, 29, 31, 36). In some tissues, the expression level of CaSR is dependent on external salinity; for example, acclimation to freshwater (FW) resulted in an increase in the mRNA expression of CaSR in the kidney of Mozambique tilapia (*Oreochromis mossambicus*) (30). A recent study in developing zebrafish has also shown that the CaSR is essential for normal skeletal development (17) and regulation of Ca\(^{2+}\) balance (26). Functional characterization of the piscine CaSR in a human embryonic kidney cell line demonstrated its sensitivity to extracellular Ca\(^{2+}\) levels, and thus it is equipped to detect alterations in Ca\(^{2+}\) levels in the external environment as well as within the body (30, 31).

Electrophysiological studies in the FW goldfish (*Carassius auratus*) revealed that the olfactory nerve increases its signalling activity with increasing environmental Ca\(^{2+}\) levels over a naturally occurring range (0.05 - 3 mM), presumably via activation of the CaSR (22). In contrast, exposure to increased water Ca\(^{2+}\) concentrations resulted in decreased firing frequency in the olfactory organ of the marine sea bream *Sparus aurata* (21). Although these different results may simply reflect species differences, it is conceivable that the functional properties of the CaSR vary between marine and FW fish.
In mammals, the CaSR exerts its Ca\(^{2+}\)-regulatory functions by modulating the secretion of calcitropic hormones (e.g. parathyroid hormone; PTH), and by its direct action in tissues involved in Ca\(^{2+}\) transport (e.g. intestine and kidney) (2). PTH is also suggested to regulate Ca\(^{2+}\) homeostasis in fish (13, 14, 26); however, the potential involvement of the piscine CaSR in the regulation of PTH remains unclear. A few previous studies on fish have demonstrated that the CaSR may regulate Ca\(^{2+}\) homeostasis through its action on stanniocalcin (STC), which is a hypocalcemic hormone that is synthesized and secreted primarily from the corpuscles of Stanni. For example, pharmacological treatment of CaSR mimetics (increase the sensitivity of CaSR) was found to stimulate the secretion of STC and decrease Ca\(^{2+}\) uptake in FW rainbow trout *Oncorhynchus mykiss* (36). Similarly, calcimimetic administration was found to increase plasma STC levels and reduced plasma concentrations of Ca\(^{2+}\) in the European flounder *Platichthys flesus* (12). It has also been suggested that the CaSR regulates whole-body Ca\(^{2+}\) balance by modulating mRNA expression of *pth-1* and *stc-1* in larval zebrafish (26).

In the gill epithelium, the CaSR is expressed in Na\(^+/K^+\)-ATPase (NKA)-rich cells (11, 29, 31), which are thought to be important, though possibly not exclusive sites of Ca\(^{2+}\) transport (33, 37). The expression of CaSR in Ca\(^{2+}\)-transporting cells suggest that the CaSR may play a role in modulating Ca\(^{2+}\) transport function in response to changing levels of environmental Ca\(^{2+}\). However, the physiological role of the CaSR in homeostatic regulation of Ca\(^{2+}\) has not been fully elucidated in fish (26).

With the above background, the potential involvement of the CaSR in Ca\(^{2+}\) homeostasis was examined in zebrafish *Danio rerio*. We hypothesized that the known ability of zebrafish to respond to low environmental Ca\(^{2+}\) levels by increasing Ca\(^{2+}\) uptake (32), is linked to the sensing ability of the CaSR. In the present study, the tissue distribution of CaSR in adult zebrafish, and
the cellular localization of CaSR in larval zebrafish were examined. Combined whole-mount in situ hybridization and immunohistochemistry were performed to evaluate the possible expression of the CaSR in ecac-expressing ionocytes. The involvement of the CaSR in Ca\(^{2+}\) homeostasis was investigated using a morpholino gene knockdown approach; Ca\(^{2+}\) fluxes and whole-body Ca\(^{2+}\) content in fish acclimated to normal- and low-Ca\(^{2+}\) water were examined in fish experiencing CaSR knockdown. Additionally, real-time PCR was conducted to evaluate the interactive effects of low-Ca\(^{2+}\) water exposure and CaSR knockdown on the mRNA expression levels of ecac, pth-1 and stc-1.

**MATERIALS AND METHODS**

*Fish*

Adult zebrafish (*Danio rerio*; Hamilton-Buchanan 1822) were purchased from Big Al's Aquarium Services (Ottawa, ON, Canada) and kept in the University of Ottawa Aquatic Care Facility, where they were maintained in plastic tanks supplied with aerated, dechloraminated City of Ottawa tap water at 28°C. The ionic composition of the water was Ca\(^{2+}\) = 0.25 mM; Na\(^+\) = 0.78 mM; Cl\(^-\) = 0.4 mM; K\(^+\) = 0.025 mM; pH 7.6. Fish were subjected to a constant 14:10-h light-dark photoperiod and fed daily until satiation with No. 1 crumble-Zeigler (Aquatic Habitats, Apopka, FL). Morpholino and sham injected embryos were reared in 50 ml Petri dishes supplemented with either dechloraminated City of Ottawa tap water or with treatment water as detailed below. The Petri dishes were kept in incubators set at 28.5°C. All experiments were performed on fish at 4-days post fertilization (dpf) except where mentioned otherwise. The experiments were conducted in compliance with guidelines of the Canadian Council of Animal
Care and after the approval of the University of Ottawa Animal Care Committee (Protocol BL-226).

**Acclimation experiments**

Control (normal) and low-Ca\(^{2+}\) water were prepared with double deionised water supplemented with CaSO\(_4\)·2H\(_2\)O, MgSO\(_4\)·7H\(_2\)O, NaCl, K\(_2\)HPO\(_4\), and KH\(_2\)PO\(_4\). The Ca\(^{2+}\) concentrations of the normal- and low-Ca\(^{2+}\) water were 250 μM and 25 μM, respectively. All other ion concentrations were kept constant (in mM): 0.8 Na\(^+\), 0.16 Mg\(^{2+}\) and 0.3 K\(^+\). Fish were transferred to control or low-Ca\(^{2+}\) water at 1 dpf, and were sampled for subsequent experiments at 4 dpf (detailed below).

**PCR analysis**

The mRNA expression of CaSR in different tissues of adults and during early developmental stages in embryos and larvae (post-hatch) was evaluated using RT-PCR. Total RNA was extracted using Trizol (Invitrogen, USA), and genomic DNA was removed with DNase I (Invitrogen, USA), following the manufacturer’s guidelines. First-strand cDNA was synthesized using RevertAid H Minus reverse transcriptase (Fermentas, CA) and random hexamer primers. PCR was conducted in 25-μl reaction volumes using 200 ng cDNA template with the following program; initial denaturation at 94°C for 30 s, followed by 30 or 40 cycles of 94°C for 30 s, 58°C for 60 s, and 72°C for 45 s, with final extension for 10 min at 72°C. Primer sets used in the present study are summarized in Table 1, and all amplicons were sequenced to ensure the correct PCR products were amplified. The housekeeping gene 18S was used as an internal control (25). The mRNA levels of CaSR in 4 dpf zebrafish following their acclimation to low-Ca\(^{2+}\) water were evaluated using real-time PCR as described previously (25). The interactive effects of low Ca\(^{2+}\)-
water exposure and CaSR knockdown on the mRNA expression of Ca$^{2+}$-transport related genes [epithelial Ca$^{2+}$ channels (ecac), Na$^+$/Ca$^{2+}$ exchanger (ncx1b) and plasma membrane Ca$^{2+}$-ATPase (pmca2)] and calciotropic hormones [parathyroid hormone-1 (pth-1) and staniocalcin-1 (ste-1)] were also examined using real-time PCR. RT-qPCR assays were performed using a Bio-Rad CFX96 qPCR system with Brilliant III SYBR Green Master Mix (Agilent Technologies, USA). All RT-qPCR was performed using the following conditions: 95°C for 3 min, 40 cycles of 95°C for 20 s and 58°C for 20 s, with final extension for 5 min at 72°C. Data were normalized to the expression of 18S, and were presented relative to the control group (sham-injected fish or fish acclimated to “normal” water).

**Western blot**

For western blot analysis, proteins from ten larvae (n=1) were extracted using an RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, 1 mM EDTA and 1 mM phenylmethanesulfonfyl fluoride) plus protease inhibitor cocktail (Roche). Samples (50 μg of protein) were loaded on a 10% SDS–PAGE and transferred to PVDF membrane (Bio-Rad). After transfer, the membrane was blocked with 5% skimmed milk in Tris buffer plus 0.05% Tween 20 (TBST) for 2 h at room temperature. The membrane was then probed with tilapia CaSR antibody (1:1000 dilution; CHIKKMGDYGDRRA) in TBST with 2% skimmed milk at 4°C overnight. The epitope of tilapia CaSR antibody is 93% identical to the zebrafish CaSR at the N-terminal (SKDQDLAARPESTQC), and the use of this antibody with zebrafish was validated in a previous study (17). After washing with TBST (three times and 5 min each; 3×5 min), the membrane was probed with 1:5000 goat anti-rabbit antibodies (Invitrogen) for 2 h at room temperature. The membrane was then washed (5×5 min) and the bands were detected using enhanced chemiluminescence (SuperSignal West femto...
chemiluminescent substrate; Pierce) with a ChemiDoc system (Bio-Rad). Subsequently, the membrane was re-probed with β-actin antibodies (1:4000; Sigma) after striping with Re-Blot Plus solution (Millipore, Billerica, MA, USA).

Whole-mount immunohistochemistry

For immunostaining of CaSR, 4 dpf larvae were first fixed with 4% paraformaldehyde in a phosphate-buffered saline (PBS) for 1 h at room temperature. After fixation, the fish were briefly rinsed with PBS with 0.1% tween (PBST), and then gradually dehydrated with 100% methanol. Following rehydration with PBST, the fish were blocked with 3% BSA for 1 h and then incubated with 1:500 dilution of CaSR antibody in PBST (plus 3% BSA and 0.8% Triton-X) at 4°C overnight. Subsequently, the fish were incubated in an Alexa 488-coupled goat anti-rabbit IgG at 1:500 dilution (Invitrogen) for 2 h in the dark at room temperature. The images were acquired using a confocal laser scanning microscopy (A1R+; Nikon Instruments Inc., USA). To determine whether CaSR was expressed in mitochondrion-rich cells, 4 dpf larvae were incubated with 50 µg/L Alexa-596 conjugated Mitotracker® (Invitrogen, Burlington, ON, Canada) for 30 min prior to fixation. The potential expression of CaSR in Na⁺/K⁺-ATPase-rich cells (NaR) was also examined by staining the fish with both CaSR and Na⁺/K⁺-ATPase (α5, diluted 1:250 in PBST; Developmental Studies Hybridoma Bank, University of Iowa) antibodies after fixation. The CaSR and Na⁺/K⁺-ATPase were then labeled with rabbit Alexa 488- and mouse Alexa 546-conjugated secondary antibodies, respectively, and images were acquired as described above.

Whole-mount in situ hybridization
A fragment of zebrafish ecac mRNA from 4 dpf larval zebrafish cDNA was PCR-amplified (Forward; 5’-TGG CTC AGG ATG CAG AAC AG-3’, reverse; 5’-TAG GGT CCC AGC ATC TCG AA-3’; size = 772 bp), cloned into a pDrive cloning vector (Qiagen, USA) and sequenced. After plasmid purification and linearization, an ecac RNA probe was synthesized by in vitro transcription in the presence of digoxigenin (dig)-UTP (Roche, Penzberg, Germany). Whole-mount in situ hybridization of larval zebrafish was performed as described previously (38), with minor modifications. In brief, 1-phenyl-2-thiourea (PTU)-treated fish at 4 dpf were fixed with 4% paraformaldehyde overnight at 4°C, and washed several times with PBST before gradual dehydration using methanol. After rehydration with PBST, the fish were permeabilized in acetone for 20 min at -20°C and then washed with PBST. The fish were first pre-hybridized in a hybridization buffer supplemented with 500 μg/ml yeast tRNA and 50 μg/ml heparin (Sigma) for 2 h at 65°C, and then incubated with 100 ng of ecac RNA probe overnight at 65°C. After serial washing with hybridization buffer and PBST, the fish were incubated in a blocking solution containing 10% calf serum in PBST for 2 h before incubating with an alkaline phosphatase-conjugated anti-dig antibody (1:2000 dilutions for 2 h at room temperature). Subsequently, the fish were washed with PBST and incubated in a NBT/BCIP staining buffer until the desired coloration intensity was obtained.

To evaluate whether CaSR and Na+/K+-ATPase were expressed in ecac-expressing ionocytes, in situ hybridization and immunohistochemistry were performed in combination. Following in situ hybridization, fish were pre-fixed in 4% PFA at room temperature for 20 min. The fish were then washed several times with PBST and then incubated overnight at 4°C with the CaSR (1:500 dilution) or Na+/K+-ATPase (1:250 dilution) antibody. The CaSR and Na+/K+-

ATPase were then labeled with rabbit Alexa 488- and mouse Alexa 546-conjugated secondary antibodies, respectively, and images were visualized as described above.

Microinjection of antisense morpholino oligonucleotide

A morpholino oligonucleotide (5’-ACT TCA GAT GAA ACC TCA TTG CTT C-3’; Genetools, OR, USA) was designed to bind to the translation start site of zebrafish CaSR (Based on the sequence information from GenBank ID XM_684005.2 and Ensembl Gene ID ENSDART00000013649). The morpholino was diluted in a Danieau buffer [58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5.0 mM HEPES (pH 7.6)] plus 0.05% phenol red before injection. A “sham” group was injected with a standard control morpholino (5’-CCT CTT ACC TCA GTT ACA ATT TAT A-3’; GeneTools) prepared as the CaSR morpholino. In preliminary trials, 2 to 6 ng of morpholino (1 nl constant volume) was injected into one-cell stage embryos using a micro-injector system (model IM 300; Narishige, Long Island, NY). Because we observed that injection of 4 ng morpholino was the optimum dose to effectively knock down the CaSR expression (see results) without inducing developmental abnormalities, this dose was used in all subsequent experiments. All experiments were performed at 4 dpf except where otherwise mentioned and only fish that had emerged from the chorion were used for experiments.

Measurement of Ca²⁺ fluxes

Following acclimation to either low- or normal-Ca²⁺ water, fish were transferred to a 2.0 ml microfuge tube and exposed to 0.2 μCi/mL ⁴⁵Ca²⁺ (as CaCl₂; American Radiolabeled Chemicals Inc., USA) for 2 h; water samples were collected at 0 and 2 h. All fluxes were performed in the normal-Ca²⁺ water. At the end of the flux period, fish were killed with an overdose of MS-222,
rinsed in isotope-free water, and three fish were pooled as one sample (N = 1). Fish were
digested with a tissue solubilizer (Solvable\textsuperscript{TM}; Perkin Elmer, USA) and later neutralized using
glacial acetic acid. The radioactivity of the digest and the water samples were measured using a
liquid scintillation counter (LS-6500; Beckman Coulter Co., Canada) following addition of
scintillation cocktail (BioSafe-II; RPI co. Mt. Prospect, IL, USA). The Ca\textsuperscript{2+} influx (pmol/fish/h)
was determined using the formula:

\[
J_{\text{in}}^{\text{Ca}} = \frac{F}{SA \times n \times t}
\]

where \(F\) is the total radioactivity counted in the fish (count per minute), \(SA\) is the specific activity
of the water (cpm/nmol), \(n\) is the number of fish, and \(t\) is the duration of the experiment in hours.

To measure the whole-body Ca\textsuperscript{2+} content, fish were killed with an overdose of MS-222,
briefly rinsed in double deionised water, and 10 fish were pooled as one sample (N = 1). The fish
were digested with 1N HNO\textsubscript{3} at 70°C for 48 h, and diluted appropriately with deionized water.
The total Ca\textsuperscript{2+} concentration was measured by flame emission spectrophotometry (Spectra AA
220FS; Varian, Palo Alto, CA), and verified using certified Ca\textsuperscript{2+} standards (Fisher Scientific).

**Statistical analysis**

All statistical analyses were performed using Sigmaplot\textsuperscript{®} (version 11.2; Systat Software, Inc.,
USA). Data were either analyzed using Student’s \(t\)-test, or two-way analysis of variance
(ANOVA; morpholino knockdown and Ca\textsuperscript{2+} treatment as two independent variables) followed
by a post-hoc Holm-Sidak test. Data were either log or square-root transformed when the
assumptions of equal variance or normal distribution were violated (determined automatically by
the statistical software). Data are reported as the means ± SEM, and \( p \leq 0.05 \) was taken as the level of significance.

RESULTS

**CaSR mRNA expression in embryos and adult zebrafish**

The results of RT-PCR analysis (following 40 cycles of amplification) suggested that the CaSR mRNA was expressed ubiquitously in all tissues examined (Figure 1A). At 30 cycles, the CaSR was detected only in the gill, intestine, liver, ovary, testis and muscle, presumably owing to its relatively higher expression levels in these tissues (data not shown). In developing zebrafish, CaSR mRNA was first detected at 3 h post-fertilization (hpf) (Figure 1B). Real-time PCR data demonstrated that CaSR expression in 4 dpf fish was significantly increased after acclimation to low Ca\(^{2+}\) water (Figure 1C).

**Immunolocalization of CaSR protein in larval zebrafish**

Western blot analysis showed that the tilapia CaSR antibody detected a single band at ~100 kDa in the lysates of 4 dpf zebrafish (Figure 2A). Immunohistochemistry and confocal microscopy were used to examine the cellular localization of CaSR in 4 dpf zebrafish larvae. Figure 2B shows an image of a larval zebrafish and regions where immunostaining of the CaSR was observed. It was found that the CaSR protein was expressed in neuromasts on the head (Figure 2C) and tail (Figure 2D), the olfactory epithelium (Figure 2E), epithelial cells on the yolk sac (Figure 2F), and corpuscles of Stannius (Figure 2G and H).

**CaSR is localized in ecac-expressing ionocytes**

To determine whether CaSR protein was expressed in ionocytes on the yolk sac epithelium, double immunohistochemistry and/or *in situ* hybridization were performed. The results showed
that CaSR protein was expressed in a subset of NKA- (Figure 3A, B and C) or Mitotracker®- (Figure 3D, E and F) positive cells. Expression of *ecac* mRNA was confined to a sub-population of NKA-positive cells (Figure 4A, B and C); CaSR protein was also observed in a subset of *ecac*-expressing cells (Figure 4D, E and F).

**Morpholino knockdown reduces the protein expression of the CaSR**

Figure 5A shows a representative western blot of the CaSR in the lysates of shams and CaSR morphants at 4 days post fertilization. Subsequent quantitative analysis revealed that the protein expression of the CaSR was significantly reduced in CaSR morphants when compared to shams (Figure 5B). Additionally, no immunostaining of the CaSR protein in the ionocytes (Figure 5C) and in other CaSR-expressing tissues were observed following the knockdown (data not shown).

**CaSR morphants acclimated to a low Ca$^{2+}$ environment exhibit a reduced ability to increase Ca$^{2+}$ uptake and a reduction in whole-body Ca$^{2+}$ levels**

Two-way ANOVA analysis revealed that there was a significant overall effects of Ca$^{2+}$ treatment and morpholino knockdown on Ca$^{2+}$ influx (both at $p < 0.001$). Acclimation to low Ca$^{2+}$ water significantly increased Ca$^{2+}$ influx in shams (Figure 6A); the impact of acclimation was markedly attenuated in the CaSR morphants. No significant interactions between water Ca$^{2+}$ treatment and morpholino knockdown on Ca$^{2+}$ influx were observed ($p = 0.2$).

Two-way ANOVA analysis suggested that Ca$^{2+}$ treatment ($p < 0.005$) but not morpholino knockdown ($p = 0.8$) significantly affected the whole-body Ca$^{2+}$ content in fish. Sham fish acclimated to low Ca$^{2+}$ water were able to defend whole-body Ca$^{2+}$ content (Figure 6B). However, the CaSR morphants acclimated to low Ca$^{2+}$ water exhibited a significant reduction in whole-body Ca$^{2+}$ content (Figure 6B). No significant interactions between water Ca$^{2+}$ treatment and morpholino knockdown on whole-body Ca$^{2+}$ content were observed ($p = 0.3$).
**CaSR morphants acclimated to a low Ca\(^{2+}\) environment exhibit a reduced ability to increase**

*ecac* mRNA expression

The effects of acclimation to low Ca\(^{2+}\) water on Ca\(^{2+}\)-transport related genes was examined using real-time PCR. The mRNA expression level of *ecac* was significantly increased in fish acclimated to low Ca\(^{2+}\) water (Figure 7A) whereas *ncx1b* and *pmca2* were unaffected. Two-way ANOVA analysis suggested that there was a significant overall effect of both Ca\(^{2+}\) treatment and morpholino knockdown on *ecac* mRNA expression (both at \(p < 0.005\)). The increase in *ecac* mRNA expression in low Ca\(^{2+}\) water was significantly reduced in the CaSR morphants (Figure 7B). A statistically significant interaction between Ca\(^{2+}\) treatment and morpholino knockdown on *ecac* mRNA expression was observed (\(p < 0.05\)).

**CaSR modulates the mRNA expression of parathyroid hormone (pth-1) and stanniocalcin (stc-1)**

Two-way ANOVA analysis revealed that both Ca\(^{2+}\) treatment and morpholino knockdown resulted in an overall significant effect on *pth-1* mRNA expression (both at \(p < 0.05\)). Acclimation to low Ca\(^{2+}\) water significantly increased the mRNA expression of *pth-1* in 4 dpf larvae (Figure 8A). The expression of *pth-1* was markedly elevated in fish experiencing CaSR knockdown, and acclimation to low Ca\(^{2+}\) water did not further increase the *pth-1* expression in the CaSR morphants. There was no significant interactions between Ca\(^{2+}\) treatment and morpholino knockdown on *pth-1* mRNA expression (\(p = 0.2\)).

Two-way ANOVA analysis suggested that both Ca\(^{2+}\) treatment and morpholino knockdown resulted in an overall significant effect on *stc-1* mRNA expression (both at \(p < 0.05\)). Fish acclimated to low Ca\(^{2+}\) water exhibited a significant decrease in the mRNA expression level of *stc-1* (Figure 8B). Expression of *stc-1* was significantly elevated in the CaSR morphants, and
was not reduced by exposure to low Ca\(^{2+}\) water. No significant interactions between Ca\(^{2+}\) treatment and morpholino knockdown on \textit{ste-1} expression were recorded (\(p = 0.1\)).

**DISCUSSION**

**Overview**

The CaSR is essential for maintaining Ca\(^{2+}\) balance in mammals (8, 18, 24), and is proposed to serve as a salinity sensor in fish (31). However, the physiological significance of the CaSR in Ca\(^{2+}\) homeostasis in fish has remained poorly understood. The results of the present study demonstrated that the CaSR is expressed in various tissues, including the olfactory epithelium, corpuscles of Stannius and \textit{ecac}-expressing ionocytes. These observations suggest that the CaSR may be involved in environmental Ca\(^{2+}\) sensing as well as regulation of Ca\(^{2+}\) transport function. Using a gene knockdown strategy, we showed that the capacity to regulate whole-body Ca\(^{2+}\) levels, increase \textit{ecac} expression and Ca\(^{2+}\) uptake was reduced in CaSR morphants acclimated to low Ca\(^{2+}\) water. The reduced regulatory ability of the CaSR morphants was associated with elevated mRNA expression of the hypocalcemic hormone, stanniocalcin. Overall, the findings of the present study suggest that in larval zebrafish, the CaSR responds to the reduced water Ca\(^{2+}\) content by increasing \textit{ecac} expression and Ca\(^{2+}\) influx, ultimately allowing the fish to maintain whole-body Ca\(^{2+}\) levels.

**Expression patterns of CaSR mRNA and localization of CaSR protein in ionocytes**

In the present study, we observed that the CaSR was expressed ubiquitously in tissues of adult zebrafish, including the gill, kidney and intestine. These findings are in consistent to recent studies in adult zebrafish (17, 26) as well as in many other teleosts [for a review, see (28)]. In larval zebrafish, we also observed that the CaSR was expressed in the olfactory epithelium. The
precise role of the CaSR in the olfactory organ is unclear, but was proposed to serve as environmental salinity sensor (31). Interestingly, we also observed that CaSR was expressed in the mechanosensitive neuromasts in larval zebrafish. A previous study showed that the plasma membrane Ca\textsuperscript{2+}-ATPase (pmca2) also is expressed in neuromasts where it may play a role in sensory organ development (9). Furthermore, parathyroid hormone (PTH) protein was observed in neuromasts in developing zebrafish (20). In mammals, the CaSR is known to regulate the secretion of PTH from the parathyroid cells (3); whether CaSR in zebrafish neuromasts is involved in environmental Ca\textsuperscript{2+} sensing and regulates the secretion of PTH requires further investigation.

Several previous studies have shown that the CaSR is abundantly expressed in the corpuscles of Stannius in fish (11, 12, 26, 29, 36). Similarly, we demonstrated that the CaSR protein is expressed in the presumed location of the corpuscles of Stannius in larval zebrafish, potentially to detect changes in internal Ca\textsuperscript{2+} levels. On the other hand, we observed that the mRNA expression of CaSR was significantly increased in larval zebrafish after acclimation to low Ca\textsuperscript{2+} water. Loretz at el. (29, 30) also reported that acclimation to freshwater (FW) increased the mRNA expression of CaSR in the kidney of Mozambique tilapia. The authors suggested that the increased renal CaSR expression could increase tissue responsiveness to internal Ca\textsuperscript{2+} levels during acclimation to FW.

The results of previous studies indicated that the CaSR is expressed in mitochondrion- and NKA-rich cells of the gill epithelium (11, 29, 31). In the present study, we demonstrated that the CaSR is expressed in a portion of NKA- and Mitotracker\textsuperscript{®}-positive cells on the skin of yolk sac in larval zebrafish. In agreement with the findings of Pan et al. (32), we observed that ecac mRNA was expressed in a subset of NKA-positive cells (also known as NaR cells) on the skin of
yolk sac in larval zebrafish. Results from double *in situ* hybridization and immunohistochemistry further revealed that CaSR is expressed in a subset of *ecac*-expressing ionocytes (i.e. NaR cells). In larval zebrafish, Ca\(^{2+}\) uptake is thought to be mediated by NaR cells on the skin of the yolk sac; the entry of Ca\(^{2+}\) across the apical membrane of these cells is facilitated by ECaC, and Ca\(^{2+}\) is subsequently extruded into the extracellular fluids via PMCA and/or NCX at the basolateral membrane (for recent review, see 23). Therefore, the expression of CaSR in NaR cells suggests a role for the CaSR in regulating Ca\(^{2+}\) transport across these cells.

**CaSR-deficient fish have a reduced ability to maintain Ca\(^{2+}\) homeostasis in a low Ca\(^{2+}\) environment**

It is well documented that fish acclimated to a low Ca\(^{2+}\) environment maintain whole-body Ca\(^{2+}\) homeostasis by increasing Ca\(^{2+}\) uptake (7, 32, 35). Pan et al. (32) reported that larval zebrafish exposed to low Ca\(^{2+}\) water increase Ca\(^{2+}\) uptake by increasing the expression of *ecac*. Similarly, we observed that the mRNA expression of *ecac* was increased in zebrafish acclimated to low Ca\(^{2+}\) water. Acclimation to low Ca\(^{2+}\) water did not significantly affect the mRNA expression of *pmca2* and *ncx1b*, supporting previous assertions that apical influx of Ca\(^{2+}\) via ECaC is the rate-limiting step (32, 34). In fish experiencing CaSR knockdown, uptake of Ca\(^{2+}\) and *ecac* mRNA were both increased following acclimation to low Ca\(^{2+}\) water; however, the increases were markedly lower when compared to control fish. Additionally, a significant reduction in the whole-body Ca\(^{2+}\) content was observed in the CaSR morphants acclimated to low Ca\(^{2+}\) water. These results suggested that fish lacking CaSR exhibit a reduced ability to increase *ecac* expression and Ca\(^{2+}\) uptake, thereby reducing their ability to maintain whole-body Ca\(^{2+}\) balance during acclimation to a low Ca\(^{2+}\) environment. Although the present study did not measure Ca\(^{2+}\) efflux, it is to be noted here that Ca\(^{2+}\) efflux is an important component for overall
Ca$^{2+}$ balance. In mammals, it has been suggested that the CaSR modulates paracellular Ca$^{2+}$ permeability in the kidney through its regulation on a tight junction protein claudin-14 (10). It remains to be examined whether the piscine CaSR is involved in the regulation of paracellular Ca$^{2+}$ movement.

In mammals, inactivation of the CaSR was reported to increase the secretion of PTH, which can ultimately increase renal reabsorption of Ca$^{2+}$ and increase plasma Ca$^{2+}$ levels (18, 40). Knockdown of the CaSR was also found to increase mRNA expression of pth-1 in 3 dpf larval zebrafish (26). Similarly, the present study also demonstrated that mRNA expression of pth-1 was significantly increased in 4 dpf CaSR morphants, potentially serving to promote Ca$^{2+}$ retention. Interestingly, however, we observed that the overall Ca$^{2+}$ influx was decreased in the CaSR morphant, presumably owing to the elevated stc-1 mRNA expression (discussed below).

Presently, the precise role of PTH in Ca$^{2+}$ homeostasis in fish is not clear (14). In larval zebrafish, overexpression of PTH1 by injecting pth-1 cRNA results in an increase in whole-body Ca$^{2+}$ content (26). Similarly, exposure to PTH-related peptide (PTHrP; structurally similar to PTH) increases Ca$^{2+}$ uptake and reduces Ca$^{2+}$ efflux in larval sea bream Sparus aurata L. (13). In contrast, injections of bovine PTH reduces plasma Ca$^{2+}$ concentration in the FW tilapia Oreochromis mossambicus and killifish Fundulus heteroclitus acclimated to a low Ca$^{2+}$ environment (1). Clearly, the involvement of CaSR in regulating PTH secretion and its subsequent action on Ca$^{2+}$ homeostasis in zebrafish, particularly in a low Ca$^{2+}$ environment, warrants further investigation.

In the present study, the precise mechanisms by which CaSR regulates Ca$^{2+}$ transport function remain unclear, but appear to be associated with the hypocalcemic hormone STC. Elevation of plasma STC levels is known to reduce Ca$^{2+}$ uptake and/or plasma Ca$^{2+}$
concentrations in fish, including European flounder and rainbow trout (12, 36). Tseng et al. (39) reported that larval zebrafish exposed to low Ca\(^{2+}\) water exhibit a reduction in \textit{ste-1} mRNA expression, and that \textit{ste-1} knockdown increases \textit{ecac} expression and Ca\(^{2+}\) influx. Consistently, we also observed that mRNA expression of \textit{ste-1} was significantly decreased in larval zebrafish acclimated to low Ca\(^{2+}\) water, presumably to increase \textit{ecac} expression and Ca\(^{2+}\) uptake. Lin et al. (26) have recently suggested that the CaSR may have time-dependent effects on the mRNA expressions of \textit{pth-1} and \textit{ste-1} in developing zebrafish. Specifically, they demonstrated that \textit{ste-1} mRNA expression in the CaSR morphants was first decreased at 30 hpf, but was then returned to the control levels at 3 dpf (26). Additionally, mRNA level of \textit{ste-1} in 3 dpf CaSR morphants was significantly increased following acclimation to either low or high Ca\(^{2+}\) water (26). Similarly, the present study demonstrated that mRNA level of \textit{ste-1} was significantly increased in the CaSR morphants after acclimation to normal or low Ca\(^{2+}\) water. In teleost, the CaSR in the corpuscles of Stannius is proposed to detect changes in internal Ca\(^{2+}\) levels and to regulate the secretion of STC (12, 36). Because the CaSR also is expressed in the corpuscles of Stannius in larval zebrafish, it is possible that knockdown of the CaSR impaired the Ca\(^{2+}\)-sensing ability in the corpuscles of Stannius, leading to the increase in \textit{ste-1} expression. In support of this idea, we observed that mRNA expression of \textit{ste-1} in the CaSR morphants, unlike in the shams, was not decreased by low Ca\(^{2+}\)-water exposure. Overall, it is likely that the inability to reduce \textit{ste-1} mRNA expression in the CaSR morphants leads to their inability to increase \textit{ecac} expression and thereby Ca\(^{2+}\) influx in the absence of appropriate sensory input from the CaSR.

\textbf{Concluding remarks and perspectives}

In vertebrates including fish, the maintenance of Ca\(^{2+}\) at appropriate levels is vital for normal physiological functions, and therefore Ca\(^{2+}\) balance must be tightly regulated. FW fish living in
naturally soft (low Ca\(^{2+}\)) water face additional challenges for maintaining Ca\(^{2+}\) balance. Although an increase in the Ca\(^{2+}\) transporting capacity of fish exposed to low Ca\(^{2+}\) environments is well-documented (7, 32, 35), the mechanism by which fish can “detect” changes in the environmental Ca\(^{2+}\) availability and thereby modulate the Ca\(^{2+}\) transport function have remained poorly understood. Here we report that the CaSR is expressed in the olfactory epithelium, corpuscles of Stannius and ecac-expressing ionocytes in larval zebrafish, implying involvement of the CaSR in environmental and internal Ca\(^{2+}\) sensing, and possible direct regulation of Ca\(^{2+}\) transport function. Additionally, knockdown of the CaSR reduced the ability of fish to increase ecac expression and Ca\(^{2+}\) uptake, and hence maintain whole-body Ca\(^{2+}\) levels, during acclimation to soft water. These results indicate that in zebrafish larvae the modulation of Ca\(^{2+}\) transport function accompanying reduced water Ca\(^{2+}\) levels, is at least in part mediated by the CaSR. Overall, results from the present study as well as from the recent study by Lin et al. (26) suggest that the CaSR is essential for maintaining Ca\(^{2+}\) balance in developing zebrafish. In mammals, the CaSR is thought to be involved in many biological processes including phosphate homeostasis and acid secretion (4, 5); it remains to be determined whether the CaSR has similar physiological functions in fish.

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Grants
REFERENCES


**Table 1:** Primer sets used for real-time PCR analysis

<table>
<thead>
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| casr | Forward: 5’-AAA TGC CCA AAC AAC TCC TG-3’  
       Reverse: 5’-GGT TTG ATG CCT TCA CGA TT-3’ | N/A*      |
| ecac | Forward: 5’-TCC TTT CCC ATC ACC CTC T-3’  
       Reverse: 5’-GCA CTG TGG CAA CTT TCG T-3’ | (27)      |
| ncx1b | Forward: 5’-TAA AGT GGC AGC GAT ACA GGT-3’  
        Reverse: 5’-CAG ATC AAG GCG AAG ATG G-3’ | (27)      |
| pmca2 | Forward: 5’-AAG CAG TTC AGG GGT TTA C-3’  
        Reverse: 5’-CAG ATC ATT GCC TTG TAT CA-3’ | (27)      |
| pth-1 | Forward: 5’-TCA TAA GCA TGT GGA GCT GAG GCA-3’  
        Reverse: 5’-ACG ATG GGT TCA TGA GCT TCT CCA-3’ | N/A*      |
| stc-1 | Forward: 5’-CCA GCT GCT TCA AAA CAA ACC-3’  
        Reverse: 5’-ATG GAG CGT TTT CTG GCG A-3’ | (39)      |

*Primer sets for *casr* and *pth-1* were designed in the present study. *casr*: Ca\(^{2+}\)-sensing receptor; *ecac*: epithelial Ca\(^{2+}\) channels; *ncx1b*: Na\(^+\)/Ca\(^{2+}\) exchanger isoform 1b; *pmca2*: plasma membrane Ca\(^{2+}\)-ATPase isoform 2; *pth-1*: parathyroid hormone isoform 1; *stc-1*: stanniocalcin isoform 1.
Figure legends

Figure 1. (A) The mRNA expression of calcium-sensing receptor (CaSR) in various tissues of adult zebrafish and (B) at different developmental stages of embryos and larvae; 18S was used as an internal control. (C) The effects of acclimation to normal (250 μM) or low (25 μM) Ca^{2+} water on the mRNA expression levels of CaSR in larval zebrafish at 4 days post fertilization (dpf). Data were normalized with 18S and were expressed relative to the fish maintained in normal Ca^{2+} water. Statistical difference (Student’s t-test, p < 0.05) is denoted with an asterisk. Data are means ± SEM, N = 6.

Figure 2. (A) A representative western blot showing that the CaSR antibody yielded a single immunoreactive band at ~100 kDa in the lysates of zebrafish larvae at 4 days post fertilization (dpf). (B) A picture of a 4 dpf larval zebrafish with arrows indicating the regions where CaSR immunostaining was observed. Fluorescent immunohistochemistry and confocal microscopy of the CaSR showing the CaSR protein expression (green) in neuromasts on (C) the head region as well as (D) on the tail. The inset image in (C) is a neuromast under higher magnification. CaSR is also expressed in the (D) olfactory epithelium, (E) ionocytes on the skin of the yolk sac, and (F, G) corpuscles of Stannius. In (F) and (G), a double immunostaining of CaSR (green) and Mitotracker® (red) was performed. Scale bars = 50 μm.

Figure 3. Double fluorescence immunohistochemical confocal images of zebrafish larvae at 4 days post fertilization (dpf), illustrating presence of the calcium-sensing receptor (CaSR; green) in ionocytes on the skin of the yolk sac. Some CaSR-positive cells were enriched with (A, B and C) Na^{+}/K^{+}-ATPase (NKA, red) or (D, E and F) mitochondria (Mitotracker® staining, red). The inset image in (F) shows membranous CaSR expression in a mitochondrion-rich cell under
higher magnification. Cells labelled with arrows represent areas of co-localization. Scale bar = 20 μm.

**Figure 4.** Double immunohistochemistry and *in situ* hybridization images of zebrafish larvae at 4 days post fertilization (dpf), illustrating presence of the calcium-sensing receptor (CaSR) in *ecac*-expressing ionocytes on the skin of the yolk sac. A portion of *ecac* mRNA was co-localized with (A, B and C) Na⁺/K⁺-ATPase (NKA) and (D, E and F) CaSR- positive cells. Scale bar = 20 μm.

**Figure 5.** (A) A representative western blot showing the protein expression of the calcium-sensing receptor (CaSR) in the lysates of shams and CaSR morphants (CaSR MO) at 4 days post fertilization (dpf). β-actin was used as an internal control. (B) Subsequent quantitative analysis revealed that the protein expression level of the CaSR in the morphants was significantly reduced following morpholino knockdown (Student's t-test; *p*<0.05). Data are means ± SEM, *N* = 3. (C) Immunohistochemistry and confocal microscopy revealed that CaSR protein was expressed in ionocytes of sham fish (cells labelled with arrows), whereas its expression was virtually absent in CaSR MO.

**Figure 6.** (A) Influx of Ca²⁺ and (B) whole-body Ca²⁺ levels in shams and calcium-sensing receptor morphants (CaSR MO) after acclimation to normal (250 μM) or low (25 μM) Ca²⁺ water. Bars labelled with different letters represent a statistical difference (two-way ANOVA, followed by a post-hoc Holm-Sidak test; *p* < 0.05). Data are means ± SEM, *N* = 6.
Figure 7. (A) The mRNA expression of epithelial Ca\(^{2+}\) channel (ecac), Na\(^+/Ca\(^{2+}\) exchanger (ncx1b) and plasma membrane Ca\(^{2+}\)-ATPase (pmca2) in 4 days post fertilization (dpf) larval zebrafish after acclimation to normal (250 \(\mu\)M) or low (25 \(\mu\)M) Ca\(^{2+}\) water. An asterisk indicates a significant difference between normal- and low Ca\(^{2+}\)-treated fish (Student’s t-test; \(p < 0.05\)). Values are means ± SEM, \(N = 6\). (B) The mRNA expression of ecac in shams and calcium-sensing receptor morphants (CaSR MO) after acclimation to normal or low Ca\(^{2+}\) water. Bars labelled with different letters represent a statistical difference (two-way ANOVA, followed by a post-hoc Holm-Sidak test; \(p < 0.05\)). Data are means ± SEM, \(N = 6\).

Figure 8. (A) The mRNA expression of parathyroid hormone-1 (pth-1) and (B) stanniocalcin-1 (stc-1) in shams and calcium-sensing receptor morphants (CaSR MO) after acclimation to normal (250 \(\mu\)M) or low (25 \(\mu\)M) Ca\(^{2+}\) water. Bars labelled with different letters represent a statistical difference (two-way ANOVA, followed by a post-hoc Holm-Sidak test; \(p < 0.05\)). Data are means ± SEM, \(N = 6\).
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.

A

Sham CaSR MO

CaSR

β-actin

B

Relative protein expression

0.0
0.2
0.4
0.6
0.8
1.0
1.2

Sham CaSR MO

C

Yolk sac

Sham CaSR MO
Figure 6.
Figure 7.

A

![Bar graph showing relative mRNA expression of ecac, ncx1b, and pmca2 under normal and low Ca²⁺ conditions.](image)

B

![Bar graph showing relative mRNA expression of ecac under different CaSR conditions.](image)
Figure 8.

A. Relative mRNA expression of *pth-1* in different conditions: Normal and Low Ca\(^{2+}\) with Sham and CaSR MO treatments. 

B. Relative mRNA expression of *stc-1* in different conditions: Normal and Low Ca\(^{2+}\) with Sham and CaSR MO treatments.