Hypothermal effects on expression of regucalcin, a calcium-binding protein, in the livers of seawater- and fresh water–acclimated milkfish, *Chanos chanos*

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Abstract Regucalcin (RGN) is a calcium-binding protein mainly expressed in the liver. It functions in regulating activities of several calcium-dependent enzymes related to energy metabolism, antioxidant mechanisms, and apoptotic pathways. Previous proteomics analyses revealed downregulation of regucalcin in milkfish livers when acclimated to low temperature (18 °C) from normal temperature (28 °C). This study first identified the full-length sequence of milkfish regucalcin from the livers with high similarity in the protein structure and calcium-binding function compared to the regucalcin of other animals. The mRNA and protein expression of regucalcin in the livers of fresh water (FW)– and seawater (SW)-acclimated milkfish under hypothermal acclimation were further analyzed. In FW milkfish, upregulation of regucalcin was found in mRNA and protein levels from 2 to 4 days, respectively, to 1 week after transfer to 18 °C for the two. However, in SW milkfish, upregulation of regucalcin occurred quickly and returned to the basal levels in 1 (mRNA expression) or 2 days (protein expression) up until 1 week after transfer. These results suggested potential roles of regucalcin in maintaining calcium homeostasis and its correlation to differential physiological responses in the livers of milkfish when they were acclimated to FW and SW.

Keywords *Chanos chanos* · Regucalcin · Liver · Low temperature · Salinity

Introduction Regucalcin was discovered in 1978 as a calcium (Ca$^{2+}$)-binding protein in the liver of rats (Yamaguchi and Yamamoto 1978) and is known as the senescence marker protein-30 (SMP30) that decreases expression with aging in rat livers (Fujita et al. 1992). Being a calcium-binding protein without EF-hand motif, several functions of regucalcin have been reported in mammals including intracellular calcium homeostasis, modulation of Ca$^{2+}$- or Ca$^{2+}$/calmodulin-dependent enzymes, participation of the biosynthesis process of ascorbic acid, and transcriptional regulation for several hormones (e.g., insulin and estrogen) (Fujita et al. 1998; Kondo et al. 2006; Yamaguchi 2011, 2013). Many vertebrates have the ability to synthesize ascorbic acid; however, this does not include teleosts due to their loss of an important key enzyme, gluconolactone oxidase (Ching et al. 2015).
As a calcium-binding protein, regucalcin was found to have inhibitory effects on several Ca\(^{2+}\)-dependent kinase and enzymes (i.e., cAMP phosphodiesterase, caspase-3, and nitric oxide synthase) which inhibited apoptosis under stress (Matsutama et al. 2004; Izumi and Yamaguchi 2004; Yamaguchi and Kurota 1997). Regucalcin knockout in mice has been reported to elevate oxidative stress, antioxidant dysfunction, and hepatocyte apoptosis, leading to liver fibrosis and even death (Park et al. 2010; Kondo et al. 2006).

Therefore, regucalcin has critical functions in calcium homeostasis for several physiological responses. Related studies on teleosts, however, are limited.

Hypothermal stress was found to change cell membrane fluidity and protein structure that may lead to rearrangement of cytoskeleton, activation of calcium channels, or ER stress–induced imbalance of cytosolic Ca\(^{2+}\) levels, which then affect several physiological responses (Bayley et al. 2018; Wang et al. 2019). Meanwhile, hypothermal stress may induce reactive oxygen species (ROS) due to the immune response or mitochondria dysfunction (Donaldson et al. 2008). Elevation of cytoplasmic Ca\(^{2+}\) levels was found in pufferfish upon low-temperature challenge, leading to oxidative stress and apoptosis (Cheng et al. 2018).

Several calcium-binding proteins were reported to be involved in mechanisms of cold tolerance in fish. The livers of the Antarctic notothenioid fish (Dissostichus mawsoni (Dm)) contained very high levels of calmodulin. Overexpression of Dm-calmodulin further demonstrated increased cold tolerance in tobacco (Na et al. 2013). In addition, feed supplemented with Dm-calmodulin recombinant protein has enhanced cold tolerance in juvenile, orange-spotted grouper (Epinephelus coioides), showing increased antioxidant enzyme activity and reduced oxidative stress responses upon low-temperature challenge (Luo et al. 2015). In the fruit fly, high expression of the Drosophila-cold-acclimation (Dca) gene, a regucalcin-like protein with the function of maintaining calcium homeostasis, was found to help the fly tolerate cold treatments (Arboleda-Bustos and Segarra 2011). A proteomic analysis revealed that the protein spot corresponding to regucalcin in seawater (SW) milkfish livers disappeared after 1-week acclimation to 18 °C (Chang et al. 2016b). Hence, it is suggested that regucalcin may play roles in maintaining calcium homeostasis in milkfish. Furthermore, the disappearance of the regucalcin protein spot in the livers of hypothermal milkfish suggested functional deterioration of regucalcin, together with the elevation of oxidative stress and dysfunction of antioxidant mechanisms (Chang et al. 2016b). In zebrafish, regucalcin was found to express mainly in the liver, corresponding to liver injury. Meanwhile, mRNA expression of zebrafish regucalcin (rgn) decreased with aging (Fujisawa et al. 2011). In rainbow trout, the strains TCO (imported strain) and BORN (regional breeding strain) revealed different patterns of rgn expression in several tissues including the liver, head kidney, and muscle. After 21-day infection with Aeromonas salmonicida, the rgn was upregulated in the liver of only the TCO strain. In addition, lower and higher temperature challenges revealed significant differences in expression of rgn between the TCO and BORN strains. There was no comparison, however, in the liver, head kidney, trunk kidney, and muscle between different temperature groups (Verleih et al. 2011). Although plasma calcium in milkfish was not changed under hypothermal challenge, calcium imbalance may happen on cellular levels according to the proteomics analysis (Kang et al. 2015; Chang et al. 2016b). Therefore, regucalcin could be considered a novel indicator of milkfish for hypothermal acclimation.

Milkfish is a tropical species with high mortality during cold snaps in winter in Southeast Asia (Fachry et al. 2018; Liao 1991; Martinez et al. 2006). The cold snap is a rapid fall of environmental temperature to be lower than 10 °C within 24 h. Being a euryhaline aquaculture species, milkfish have the ability to survive in environments with a broad range of salinities and thus have been cultured in water with different salinities (Jana et al. 2006). In previous studies, SW-acclimated milkfish showed better low-temperature tolerance than fresh water (FW)–acclimated milkfish (Kang et al. 2015). When acclimated to low-temperature environments, different strategies in energy metabolism and antioxidant mechanisms were found in the livers of FW- and SW-acclimated milkfish (Chang et al. 2016a, 2017, 2018, 2019). Since regucalcin, a calcium-binding protein, has regulatory functions for energy metabolism, antioxidant mechanisms, and apoptosis in mammals (Fukaya and Yamaguchi 2004; Yamaguchi 2013; Vaz et al. 2016), the regucalcin in the liver of milkfish was thought to be an upstream regulator for differential physiological responses under salinity and hypothermal conditions.
acclimation. Hence, this study reported the molecular characteristics, gene expression, and relative protein abundance of regucalcin in the livers of FW- and SW-acclimated milkfish to illustrate a potential mechanism of modulating cellular calcium for hypothermal acclimation.

**Materials and methods**

**Fish and experimental conditions**

Juvenile milkfish (*Chanos chanos*; total length: 9–10 cm, weight range: 10–12 g) were obtained from a local fish farm in Changhua, Taiwan. Fish were acclimated and maintained in 400-L tanks containing FW and SW (35‰). Milkfish were maintained in a 12-h light/12-h dark photoperiod at 28.0 ± 0.5 °C. The experimental SW was prepared from local tap water and Blue Treasure Sea Salts (New South Wales, Australia). The water was continuously recirculated through fabric-floss filters and partially replaced every month. The fish were fed daily with commercial pellets (Fwusow Industry, Taichung, Taiwan). The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Chung Hsing University, Taichung, Taiwan (IACUC Approval No. 105-024 to THL).

The cooling system was comprised of two 100-L tanks; one 100-L filter tank, and one tank fitted with an A/C compressor (PF-225M, Prince, Tainan, Taiwan) that had an electronic temperature controller. The temperature of the hypothermal treatment as 18 °C was determined according to our previous study on the nonlethal cold tolerance temperature of milkfish (Chang et al. 2016b). Milkfish were transferred to the cooling tanks with FW or SW for at least 2 days prior to beginning the hypothermal experiments to reduce the handling stress. Then, the water temperatures in the cooling tanks were lowered at a constant rate (2 °C h⁻¹) from 28 to 18 °C. Milkfish were kept at 18 °C for 1 week before sampling (*n* = 8; 2 individuals in each tank, total 4 tanks with the same experimental conditions). For the hypothermal time-course experiments, the milkfish were sampled after hypothermal treatment (18 °C) for 1, 3, 6, 12, 24, 48, 96, and 168 h (*n* = 6; 2 individuals in each tank, a total of 3 tanks with the same experimental conditions).

Total experimental individuals are 140. All experimental fish were anesthetized with 0.5% 2-phenox- yethanol before sampling. The livers of milkfish were dissected quickly, immersed in liquid nitrogen, and stored at −80 °C for the following analyses.

**Total RNA extraction and cDNA preparation**

The method used for RNA isolation was modified by Chang et al. (2016a). The RNA samples were extracted using a TRI reagent (Molecular Research Center, Cincinnati, OH, USA). All samples were homogenized using a Pellet Pestle® Cordless Motor (Kimble Chase, Millvale, NJ, USA). The quality of the extracted total RNA was determined by the NanoDrop 2000 (Thermo, Wilmington, CA, USA) and was evaluated by 1.0% agarose gel electrophoresis. One microgram of total RNA was used for cDNA synthesis using iScript™ Reverse Transcription Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s protocol. The templates of cDNA for full-length sequence cloning were obtained using the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA) following manufacturer’s instructions.

**Sequence cloning, characterization, and analysis**

Primers were designed by Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) based on a highly conserved region compared with several sequences of teleosts. The PCR products were subcloned into the pGM-T vector (GeneMark, Taipei, Taiwan) and sequencing was conducted. The full-length sequence was obtained following RACE manuscript’s instructions. All primers are listed in Table 1. The open reading frame of *rgn* was predicted by ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The sequence prediction analyses were obtained with DTU Bioinformatics (http://www.cbs.dtu.dk/services/), ExPASy (https://www.expasy.org/), Uniprot (https://www.uniprot.org/), and SWISS-MODEL (https://swissmodel.expasy.org/). The phylogenetic tree was constructed using Mega-X using the maximum likelihood method with 1000 bootstraps.
Real-time PCR

The qPCR was performed on a MiniOpicon real-time PCR (Bio-Rad). The qPCR mixture contained 8 μL of cDNA (100-fold dilution), 2 μL of 2 μM primer pairs, and 10 μL of 2× KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA). The qPCR process was conducted using the following protocol: activation step (95 °C) for 10 min, 40 cycles of 10-s denaturation step (95 °C), an annealing/extension step (60 °C) for 30 s, and a melting curve analysis from 70 to 95 °C. The qPCR primers were qualified by amplification efficiency, melting curve, single product in electrophoresis, and sequencing. Glyceraldehyde 3-phosphate dehydrogenase (gapdh) was analyzed as the normalization gene in the present experiment. The relative expression was obtained using the formula:

$$2^{-(C_{\text{rgn}, n} - C_{\text{gapdh}, n}) - (C_{\text{rgn}, c} - C_{\text{gapdh}, c})},$$

where “Ct” was the threshold cycle number, “n” indicated each sample, and “c” indicated the control mixed with cDNA samples of all experiments.

Western blot analysis

For immunoblotting analysis, the milkfish liver was placed into a 2.0-mL microcentrifuge tube and a SEID buffer was added (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, 0.1% sodium deoxycholate, pH 7.5) containing a protease inhibitor (vol/vol: 25:1; Roche, Mannheim, Germany). Then, the sample in the tube was homogenized using a Polytron PT1200E homogenizer (Lucerne, Switzerland) on ice. The supernatant concentration of the homogenate was determined using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). All samples were heated at 60 °C for 15 min with a 6× protein sample buffer (0.5 M Tris-Cl, 40% glycerol, 10% sodium dodecyl sulfate, 9.3% DTT, 0.0012% bromophenol blue, pH 6.8). One-hundred microgram denatured protein samples were fractionated by 10% polyacrylamide gel. Gels were electrophoresed and transferred to 0.45 μm PVDF membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% (w/v) nonfat dried milk in PBST (phosphate-buffered saline with 0.1% Tween-20) for 1 h. PVDF membranes were subsequently incubated with the SMP30 antibody (sc377184, Santa Cruz Biotech., Dallas, TX, USA) or GAPDH antibody (GTX100118; GeneTex, Irvine, CA, USA) overnight at 4 °C. Regucalcin (0.2 μg mL⁻¹) and GAPDH (0.1 μg mL⁻¹) antibodies were diluted with 1% bovine serum albumin (CyrusBioscience, Taipei, Taiwan) in PBST. The secondary antibody (goat anti-rabbit IgG antibody HRP, GTX213110-01, GeneTex; rabbit anti-mouse IgG antibody HRP, GTX213112-01, GeneTex) was incubated at room temperature for 1 h. The Western chemiluminescence HRP substrate (T-Pro Biotechnology, Taipei, Taiwan) was used to develop the immunoblots. Images were photographed with a cool-charge-coupled device (CCD) camera (ChemiDoc XRS+, Bio-Rad) and analyzed using Image Lab v. 3.0 software (Bio-Rad). The intensity of immunoreactive bands was calculated to numerical values and normalized against GAPDH. The PVDF membrane was reused one-time for GAPDH antibody loading control, by added striping buffer (63 mM Tris-HCl, 70 mM sodium dodecyl sulfate, pH 6.8) with 0.7% β-mercaptoethanol (Millipore) at 50 °C for 30 min and re-blocked following the method described above.

Statistical analysis Values were expressed as means ± SEM (standard error of the mean). Data on tissue distribution and hypothermal time-course experiments were compared using a one-way
ANOVA with Tukey’s and Dunnet’s test, respectively. Data from the hypothermal experiments were compared with a two-way ANOVA with Tukey’s HSD post hoc methods. All experimental data analysis was conducted using R software (R Foundation, Vienna, Austria). Significant differences were accepted when $P < 0.05$.

Results

Identification and characterization of regucalcin in milkfish

According to the transcriptomic library of milkfish and PCR cloning, the regucalcin gene was identified in milkfish (Fig. 1A). The full-length cDNA of regucalcin (MT218432) contained 1603

Fig. 1 The characteristics of milkfish regucalcin sequence. (A) The full-length nucleotide sequence and predicted amino acid sequence of milkfish regucalcin. Gray box: β-strand; red box: α-helix. (B) The predicted protein structure of milkfish regucalcin. Four residues (E18, N103, N151, and D201) are predicted to be the calcium-binding residues (green spot). (C) Phylogenetic analysis of the amino acid sequence of milkfish regucalcin. The phylogenetic tree was constructed using the maximum likelihood method, and the number indicates bootstrap values for 1000 replicates. The accession numbers of different species are listed in Supplementary Table 1.
bp including an ORF of 891 bp, a 5′-UTR and 3′-UTR of 79 bp and 821 bp, respectively. The ORF encoded 296 amino acids with a molecular weight of 33 kDa and theoretical isoelectric point (pI) of 5.59. The sequence was predicted to have one phosphorylation site at S14 and no signal peptide. The predicted protein structure of regucalcin with calcium dock-site (green spot) exhibited the calcium-binding residues (E18, N103, N151, and D201; Fig. 1B) and the gluconolactonase (GNL) [EC 3.1.1.17] region (V9-S261).

Phylogenetic tree analysis and tissue distribution of regucalcin expression

The phylogenetic analyses revealed that milkfish regucalcin (RGN) was highly similar in the branch of Ostariophysi including channel catfish (Ictalurus punctatus; 85.6%), goldfish (Carassius auratus; 83.5%), and zebrafish (Danio rerio; 86.1%). According to different branches of subclasses, the similarities ranged from 71 to 82% compared to other teleosts (Fig. 1C). The genome sequence of milkfish was compared with other teleosts (Fig. S1). The expression of regucalcin gene (rgn) was detectable in all analyzed tissues. The highest expression of rgn was found in the liver followed by the intestine (Fig. 2).

Effects of hypothermal treatments on hepatic regucalcin expression in SW- and FW-acclimated milkfish

Under 1-week hypothermal acclimation, the hepatic rgn was upregulated in FW-acclimated milkfish and downregulated in SW-acclimated milkfish. The rgn expression in the livers of the FW/18 °C group was significantly higher than the SW/18 °C group (Fig. 3). Two-way ANOVA analyses revealed that rgn expression was affected by the hypothermal treatment ($F_{1,23} = 9.92$, $p = 0.01$) and environmental salinity ($F_{1,23} = 20.12$, $p = 0.01$). Synergistic interaction effects of hypothermal stress and salinity significantly affected the expression of rgn (Fig. 3). Upon hypothermal challenge, hepatic rgn expression was significantly upregulated at 1 h and from 48 h to 1 week after transfer to 18 °C in the FW group (Fig. 4A). On the other hand, rgn expression was found to increase significantly at 3, 6, and 12 h and return to normal level from 24 h to 1-week post transfer in the livers of SW-acclimated milkfish (Fig 4B).
Fig. 3 Expression of hepatic rgn in fresh water (FW)– and seawater (SW)-acclimated milkfish under normal (28 °C) and hypothermal (18 °C) temperatures. Different letters in the FW milkfish (a, b) and SW milkfish (a', b') indicate significant differences between the normal and low-temperature group. The x and y indicate significant differences between the FW and SW groups at 28 °C or 18 °C (P < 0.05, two-way ANOVA, Tukey’s HSD pairwise comparison). Values are means ± SEM, n = 8. T, temperature; S, salinity.

Fig. 4. The time-course expression of hepatic rgn in (A) fresh water–acclimated milkfish and (B) seawater-acclimated milkfish upon hypothermal challenge. The asterisks (*) indicate significant differences between various time points and 0 h (P < 0.05, one-way ANOVA, Dunnet’s test). Values are means ± SEM, n = 6.
The protein profiles of hepatic regucalcin under hypothermal acclimation

The single immunoreactive band of regucalcin in the milkfish liver was detected at 35 kDa, and GAPDH was used as the loading control for normalization. Under hypothermal acclimation, the protein abundance of regucalcin in FW-acclimated milkfish livers was upregulated, but in SW-acclimated milkfish, there was no significant difference. In addition, relative abundance of RGN in the livers of the FW/18 °C group was significantly higher than that of the SW/18 °C group (Fig. 5). Two-way ANOVA analyses revealed synergistic interaction effects ($F_{1,23} = 23.25$, $p = 0.03$) between the hypothermal treatment and ambient salinities on protein abundance of RGN. The factors of the low temperature ($F_{1,23} = 13.05$, $p = 0.23$) and environmental salinities ($F_{1,23} = 3.25$, $p = 0.15$), however, were not significantly different between FW- and SW-acclimated milkfish (Fig. 5). Upon hypothermal challenge, RGN protein abundance of FW milkfish livers increased gradually from 12 h after transfer to 18 °C and became significantly elevated from 96 h at 18 °C (Fig. 6A). In the SW groups, relative amounts of hepatic RGN were increased from 1 h after transfer to 18 °C, became significantly upregulated at 3 and 24 h, and returned to normal level after 48 h to 1 week at 18 °C (Fig. 6B).

Discussion

The full-length regucalcin sequence of the milkfish was cloned in the present study. The predicted protein structure was highly similar (76.3%) to human regucalcin with multiple functions including GNL for use in vitamin C biosynthesis pathway and calcium chelator for use in calcium homeostasis (Chakraborti and Bahnson 2011). However, teleosts cannot synthesize vitamin C due to the lack of a required enzyme, gulonolactone oxidase, in the biosynthesis pathway (Linster and Schaftingen 2007; Ching et al. 2015). In addition, no genetic information regarding gulonolactone oxidase can be found in the genome database.

![Fig. 5](image_url) Relative protein abundance of hepatic regucalcin (RGN) in fresh water (FW)- and seawater (SW)-acclimated milkfish at normal (28 °C) and hypothermal (18 °C) temperatures. The representative immunoblots of RGN showed a single immunoreactive band at 35 kDa. GAPDH was used as the loading control. Different letters (a, b) indicate significant differences between the 28 °C and 18 °C groups in FW or SW. The x and y indicate significant differences between the FW and SW groups at 28 °C or 18 °C ($P < 0.05$, two-way ANOVA, Tukey’s HSD pairwise comparison). Values are means ± SEM, $n = 8$. T, temperature; S, salinity

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for milkfish (http://140.120.209.83/CCD/index/C. chanos_home.htm). Regucalcin in teleosts should play major roles in maintaining calcium homeostasis and modulating calcium-dependent physiological responses. The residues, E18, N103, N151, and D201, in milkfish regucalcin sequence were highly conservative compared to the calcium-binding residues of human regucalcin (Kondo et al. 2006; Chakraborti and Bahnson 2011). The amino acid sequence of milkfish regucalcin was also highly similar (72–86%) to that of the other teleosts with conserved calcium-binding residues.

To date, there are few studies on teleostean regucalcin. The present study revealed that *rgn* was...
mainly distributed in the liver. In zebrafish, regucalcin was also mainly expressed in the liver (Fujisawa et al. 2011). In rainbow trout, however, regucalcin was found mainly in the liver and kidneys (Verleih et al. 2011). Expression levels of regucalcin in zebrafish decreased with aging which might be due to the deteriorated ability of maintaining calcium homeostasis (Fujisawa et al. 2011). A previous proteomics study on the livers of SW-acclimated milkfish identified regucalcin and found the corresponding protein spot disappeared in two-dimensional gel (2D-gel) electrophoresis under hypothermal acclimation (Chang et al. 2016b). The immunoblots in the present study, however, showed that relative abundance of regucalcin in the livers of the SW/18 °C group did not decrease significantly compared to the SW/28 °C group. A change in the isoelectric point of the protein via phosphorylation or glycosylation might be the reason that the corresponding protein spot of regucalcin disappeared in 2D-gel electrophoresis but relative protein abundance of regucalcin remained high in the livers of SW-acclimated milkfish under hypothermal acclimation.

Ca²⁺ is a common second messenger for cells (Das et al. 2018). In mammals, the calcium signal pathway is highly correlated to cold tolerance ability and affects several physiological responses including apoptosis, antioxidant capacity, and transcriptional regulator mechanisms (Kondo et al. 2006; Yamaguchi 2011). In pufferfish, the concentration of free Ca²⁺ in the cytoplasm was upregulated at lower temperatures, leading to apoptotic responses (Cheng et al. 2018). Regucalcin was reported to play an important role in regulating cytoplasmic free-Ca²⁺ levels and inhibiting excess Ca²⁺ influx to prevent apoptosis and suppress glycolytic metabolism in rats (Fujita et al. 1998; Vaz et al. 2016). Therefore, regucalcin may play similar roles in modulating Ca²⁺ in teleosts. Under hypothermal acclimation, the proteomics analysis revealed upregulation of regucalcin protein in the hepatopancreas of the white shrimp, Litopenaeus vannamei (Fan et al., 2016). The homologue of regucalcin in Drosophila was found to be upregulated during cold acclimation and was suggested to play an important role in cold tolerance ability (Goto 2000; MacMillan et al. 2016). On the other hand, calmodulin, the other calcium-binding protein, showed very high levels in the livers of the Antarctic fish (D. mawsoni). Overexpression of calmodulin in tobacco enhanced cold tolerance ability of the Antarctic fish (Na et al. 2013). In this study, milkfish raised in FW and SW showed differential profiles of regucalcin expression in both mRNA and protein levels upon hypothermal challenge. In FW-acclimated milkfish, the mRNA level was upregulated after 2 days and protein levels became significantly higher from 4 days to 1 week after transfer to 18 °C. In SW-acclimated milkfish, mRNA expression was upregulated and returned to the basal level in 1 day, and for protein expression, it was upregulated in 2 days and until 7 days after the transfer to 18 °C. Unlike the FW-acclimated milkfish, the regucalcin-involved physiological responses in SW-acclimated milkfish livers seemed to be consistent under hypothermal acclimation and the expression levels returned in 1 day at low temperatures. Kang et al. (2015) reported that SW-acclimated milkfish have a better cold-tolerant ability than FW-acclimated individuals. Although plasma calcium concentration in FW-acclimated milkfish was not changed during hypothermal acclimation (Kang et al. 2015), cellular calcium concentration in the livers of FW-acclimated milkfish might be imbalanced upon acute hypothermal challenge (Kang et al. 2015), like those found in pufferfish (Cheng et al. 2018). Imbalanced calcium contents might be one of the reasons of induced apoptosis of hepatocytes in milkfish with low-temperature treatments (Izumi and Yamaguchi 2004; Yamaguchi 2013; Chang et al. 2018; Cheng et al. 2018; Chang et al. 2021). In addition, it is possible that in milkfish livers, the fluctuation in calcium contents led to degradation of hepatic glycogen to provide energy for maintaining normal physiological responses, as was found in mice (Oliva-Vilarnau et al. 2018). On the other hand, the same patterns of regucalcin expression in transcriptional and translational levels in the livers of SW-acclimated milkfish upon hypothermal challenge suggested that SW milkfish have the ability to maintain cellular calcium homeostasis under hypothermal acclimation.

In previous mammalian studies, regulation of hepatic regucalcin expression was demonstrated by administration of calcium ions. The fluctuation in calcium levels affected MAPK kinase, protein kinase C, and calmodulin kinase, as well as activated binding of transcription factors (AP-1, NF1-A1, and RGRPP117) to specific binding sites in the sequence of regucalcin to modulate its gene expression (Murata and Yamaguchi 1998; Yamaguchi 2011). In addition, regucalcin is suggested to be a transcription
factor for regulating expression of downstream genes in mice (Yamaguchi 2013). Since the sequence of regucalcin was predicted to contain several binding sites of transcription factors (i.e., AP-1, β-catenin, C/EBP) as well as the calcium-binding sites (i.e., E18, N103, N151, and D201), the transcription activities of different calcium signaling pathways were supposed to be modulated by regucalcin in milkfish as in mammals.

Conclusions

In this study, the full-length cDNA sequence of regucalcin in milkfish (GenBank acc. MT218432) was identified and suggested to play roles for maintaining homeostasis of cellular calcium. SW-acclimated milkfish has better cold tolerance ability than FW-acclimated individuals (Kang et al. 2015). The regucalcin expression found in this study suggested that the ability for maintaining calcium homeostasis in the livers was also better in SW-acclimated milkfish rather than FW ones under an acute phase of hypothermal acclimation. The molecular evidence in this study further illustrates that salinity is an important factor that affects the maintenance of homeostasis in the euryhaline milkfish under hypothermal challenge.

Author contribution C.H.C conceived and designed this study and contributed to manuscript writing. T.H.L. reviewed and edited the manuscript. T.H.L. supervised the project. All authors read and approved the final manuscript for publication.

Funding This work was partly supported by a grant to T.H. Lee from the Ministry of Science and Technology (MOST) of Taiwan (105-2313-B-005-027-MY3 and 108-2313-B-005-006-MY3) and in part by the integrative Evolutionary Galliform Genomics (iEGG) and Animal Biotechnology Center from The Feature Area Research Center Program within the framework of the Higher Education Sprout Project by the Ministry of Education (MOE) in Taiwan (109-S-0023-F). C.H.C. was supported by the postdoctoral fellowships from the MOST (108-2811-B-005-520).

Data availability Not applicable

Code availability Not applicable

Declarations

Ethics approval The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Chung Hsing University, Taichung, Taiwan (IACUC Approval No. 105-024 to THL).

Consent to participate Not applicable

Consent for publication All authors read and approved the final manuscript for publication.

Competing interests The authors declare no competing interests.

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