Hypothermal stress induced differential expression profiles of the immune response gene, warm-temperature-acclimation associated 65-kDa protein (Wap65), in the liver of fresh water and seawater milkfish, *Chanos chanos*

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**ABSTRACT**

The milkfish (*Chanos chanos*), an important aquaculture species, is intolerant to cold environments. Temperature fluctuations in the environment affect the physiological response, behavior, and survival rate of the fish. The warm-temperature-acclimation associated 65-kDa protein (Wap65) of teleosts was identified after heat shock treatment and has two isoforms. Both the isoforms were involved in the induction of immune responses in fish. They showed high degree of sequence conservation with the mammalian hemopexin and had high affinity for heme, which helped in the neutralization of free-heme and its transport to the liver. In this study, we isolated and characterized the two isoforms of wap65 genes (*Ccwap65-1* and *Ccwap65-2*) from the liver of milkfish. The *Ccwap65-1* and *Ccwap65-2* are mainly expressed in livers of milkfish. In hypothermal treatment, the expression levels of *Ccwap65-2* in the livers of SW and FW milkfish were up-regulated after exposure to low temperature (18°C to 14°C) for 12 h and 96 h compared to those in the normal temperature (28°C to 24°C) group, respectively. After intraperitoneal injection of lipopolysaccharide (LPS), the expression of *Ccwap65-2* was elevated in both SW and FW milkfish, whereas that of *Ccwap65-1* was not affected in both the groups. Thus, *Ccwap65-2* expressed in the milkfish liver under hypothermal stress was identified as a novel immune biomarker. In addition, according to the transcriptome database, up-regulation of the other immune-response genes indicated increased pathogen infection status under hypothermal stress. Acute increase in the expression of hepatic *Ccwap65-2* in response to pathogen infection might lead to better cold tolerance of SW milkfish compared to that of the FW individuals upon cold challenge.

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slightly with fluctuations in temperature, whereas Wap65-2 responded strongly to increase in temperature and pathogen infection in hepatocytes of the turbot (*Scophthalmus maximus*), Kungfai fat minnow (*Rhynchocypris kungangensis*), and rock-bream (*Oplegnathus fasciatus*) [5,6,9,10].

Because fishes are ectothermic, changes in water temperature directly affect their physiological responses [17,18]. In winter, sudden drop in water temperatures sometimes cause the “winter syndrome” of the gilthead sea bream (*Sparus aurata*), affecting their health and increasing their mortality [18]. The “winter syndrome” is caused by several factors, including fasting, metabolic depression, ionic imbalance, and immune suppression. With lower immune capacity, the fish have higher risks of infection [18]. In the mouse model, blood degradation products (heme or hemoglobin) induce synergistic inflammation upon infection. The levels of pro-inflammatory factors, like tumor necrosis factor (TNF), interleukin (IL)-6, and toll-like receptors (TLR) are also induced. In addition, Hpx, which also functions as a TLR-agonist, was found to be capable of reducing the inflammatory response [2].

Milkfish (*Chanos chanos*) is one of the crucial economic teleosts in the Southeast Asia and Taiwan. Milkfish have high euryhalinity and can be cultured in fresh water (FW), brackish water (BW), or seawater (SW) environments [19]. In winter, high mortality of milkfish during cold snap usually causes huge economic loss in Taiwan. SW-acclimated milkfish, however, exhibit better cold tolerance than the FW individuals [20]. Based on the results of next-generation sequencing (NGS) of their transcriptome, SW milkfish were found to increase their energy budget by up-regulating the aerobic metabolism-related genes whereas FW-acclimated milkfish down-regulated the basal metabolism-related genes to reduce their energy loss [21]. Moreover, profiles of the hepatic proteomes in hypothermal SW milkfish revealed that the individuals experienced oxidative stress, which caused apoptosis. The analysis of the hepatic proteome profiles in milkfish also helped in identifying the hemopexin-like protein (Wap65) under cold stress, for the first time [22].

Earlier studies have demonstrated the expression of Wap65 in several teleosts under heat shock or pathogen infection. The present study, based on previous proteomic identification of Wap65 in SW milkfish upon cold challenge [22] and physiological findings of salinity-dependent cold tolerance in milkfish [20], aimed to identify the two isoforms of Wap65 (Ccwap65-1 and Ccwap65-2) in milkfish livers and to compare their responses to hypothermal stress in SW and FW. Moreover, we evaluated the potential roles of Ccwap65 in reducing the inflammatory responses in SW and FW milkfish by administering lipopolysaccharide injection. The effects of salinity and cold on the expression of some immune-response genes were also determined for comparison.

## 2. Materials and methods

### 2.1. Experimental fish

The juvenile milkfish were purchased from a local fish farm (Changhua, Taiwan). The experimental fish were maintained in tanks filled with 400 L fresh water (FW) or seawater (SW; 35‰), that was continuously circulated through fabric-floss filters. SW was prepared from the local tap water by adding appropriate amounts of RealOcean™ Synthetic Sea Salt (Camrillo, CA, USA). The milkfish were raised at 28 ± 1 °C with a 12:12 h light/dark photoperiod for at least one month. All experimental fish were fed with commercial pellets daily. The experimental groups including the SW- and FW-acclimated milkfish were subjected to increased cooling by reducing the temperature at a rate of 2 °C/h (from 28 °C to 18 °C) using a cooling system (PF-225M, PRINCE, Tainan, Taiwan). The body weight and total length of experimental fish were measured before sampling. The average body weight was 10.4 ± 0.5 g and average total length was 9.3 ± 0.1 cm. The mRNA expression levels of wap65 in various tissues, including brain, gill, intestine, kidney, liver, muscle, and spleen of SW milkfish at normal temperature, were analyzed by the quantitative real-time PCR. Subsequently, in the one-week hypothermal experiments, the livers of milkfish of 18 °C and 28 °C groups were sampled at 7 days, respectively, for the following analyses and comparisons. On the other hand, milkfish of the 18 °C group were sampled at 1, 3, 6, 12, 24, 48, 96, and 168 h post-cooling, for comparisons of the acute-phase changes in livers in response to hypothermal challenges between FW and SW. Six individuals from each time point were used in the one-week and acute-phase experiments. Before sampling, the fish were anesthetized with 0.5% 2-phenoxyethanol, then sacrificed by cutting their spinal cord. The protocol describing the experiments of fish was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC Approval No. 104-126R to THL).

### 2.2. Obtaining the cDNA sequence

Total RNA samples were extracted from the liver of *C. chanos* using Tripure isolation reagent (Roche, Mannheim, Germany). The genomic DNA contamination in the RNA preparation was eliminated using the RNA cleanup protocol provided with the RNAprep Mini RNA isolation kit (GE Healthcare, Piscataway, NJ, USA). The quality of the extracted total RNA was determined using NanoDrop 2000 (Thermo, Wilmington, CA, USA) and was visually assessed using electrophoresis, following the methods described in Ref. [23]. The first-strand cDNA was synthesized from 1 μg of total RNA using iScript™ Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer’s instructions.

### 2.3. Cloning of full-length *Ccwap65*-1 and *Ccwap65*-2 cDNAs from milkfish liver

The partial DNA sequences with homology to wap65-1 and wap65-2 were identified from the milkfish NGS database (Hu et al., 2015). The cDNA template for rapid amplification of cDNA ends (RACE) was made from the total RNA extracted from the liver of milkfish using SMART RACE amplification kit (Clontech, Mountain View, CA, USA). For PCR amplification, 2 μL of 5' - or 3' - RACE-cDNA was used as a template in a 50 μL reaction containing 0.25 mM dNTPs, 2.5 U EX-Taq polymerase (Takara, Shiga, Japan), and 0.2 μM of each primer. The specific primers for 5' - and 3' - RACE were designed according to the sequence of the conserved regions listed in Table 1. The RACE products were cloned into pGM-T vector (Genemark, Taipei, Taiwan) and the amplicons were confirmed by sequencing. BLAST (http://www.ncbi.nlm.nih.gov/) was used to identify the sequences of *Ccwap65*-1 and *Ccwap65*-2.

### 2.4. Sequence analysis

The cleavage sites for the signal peptide were predicted using SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). The open reading frame (ORF) of *Ccwap65*-1 and *Ccwap65*-2 were predicted with ORF finder (http://www.ncbi.nlm.nih.gov/orf/gorf.html). The molecular mass and theoretical pI were predicted using pl/Mw program (http://web.expasy.org/compute_pi/). The multiple alignment of amino acid sequences was also performed (accession numbers of the amino acid sequences of other fish and vertebrates are listed in Supplementary Table 1). The phylogenetic tree was constructed using Mega 6 by neighbor-joining method with 1000 bootstraps.
and hypothermal groups of SW and FW milkfish have been reported [21]. The link of the transcriptome database (http://140.120.209.83/CCD/index/C.chanos_home.htm) has also been published recently [24].

### 2.8. Statistical analysis

The quantitative values from the long-term expression in milkfish were compared using two-way ANOVA followed by the Student’s t-test post-hoc method. The results of the acute-phase experiments were compared using one-way ANOVA with Dunnett’s pairwise method. The LPS injection experiments were analyzed by Student’s t-test. The values were expressed as means ± SEM, and p-value < 0.05 was set as the significance level.

### 3. Results

#### 3.1. Identification and characterization of Ccwap65 cDNA isoforms and determination of their amino acid sequences

The cDNA of Ccwap65-1 consisted of a 44-bp 5’-untranslated region (UTR), 1287-bp of a single ORF encoding a polypeptide of 428 amino acids, and a 463-bp 3’-UTR (Fig. 1a; Accession number, KX421528). The Ccwap65-2 cDNA included a 75-bp 5’-UTR, 1203-bp ORF (450 amino acids), and 147-bp 3’-UTR (Fig. 1b; Accession number, KX421529). The calculated molecular weights of Ccwap65-1 and Ccwap65-2 polypeptides were 48.8 and 45.6 kDa and the theoretical pl values were 5.72 and 5.87, respectively. The predicted cleavage site of the Ccwap65-1 protein was between amino acid 16 and 17 (Fig. 1a). Signal peptide was not present in the deduced Ccwap65-2 protein (Fig. 1b). The predicted Ccwap65-1 and Ccwap65-2 proteins exhibited 5 and 4 hemopexin (Hpx) domains, respectively (Fig. 1).

#### 3.2. Phylogenetic analysis

The phylogenetic analysis (Fig. 2) revealed that the mammalian Hpx and teleostean Wap65 can be divided into three subgroups, namely Wap65-1, Wap65-2, and Hpx subgroups. The two Wap65 milkfish sequences were directly identified, which branched into each subgroup. The values for similarity/identity in the amino acids of Ccwap65-1 and Wap65-1 of the Japanese sea bass (Lateolabrax japonicus), European seabass (Dicentrarchus labrax), and Channel catfish (Ictalurus punctatus) were 80.5/52.3, 79.6/53.0, and 77.5/51.7%, respectively. Ccwap65-1 displayed similarity/identity values of 84.4/65.3, 82.9/61.7, and 82.6/63.0 at the amino acid level with the corresponding proteins of Channel catfish (I. punctatus), European seabass (D. labrax), and Japanese sea bass (L. japonicus), respectively. The sequence similarity/identity of CcWap65-1 and CcWap65-2 proteins exhibited 5 and 4 hemopexin (Hpx) domains, respectively (Fig. 1).

#### 3.3. mRNA expression profiles of Ccwap65-1 and Ccwap65-2 in the tissues of milkfish

The qPCR was used to investigate the mRNA expression of Ccwap65-1 (Fig. 3a) and Ccwap65-2 (Fig. 3b) in various tissues of the SW-acclimated milkfish. The mRNA expression levels were normalized to those of Ccgapdh2. Ccwap65-1 was expressed in the brain, gill, liver, muscle, and spleen, while Ccwap65-2 was expressed in the brain, gill, kidney, liver, and muscle. In addition, both Ccwap65-1 and Ccwap65-2 were found to express mainly in the liver, while the expression of Ccwap65-1 was 1.5 folds higher than Ccwap65-2 in livers of SW milkfish.
3.4. Effects of hypothermal and salinity treatments on the expression of hepatic Ccwap65 mRNA

The effects of hypothermal and salinity treatments on the expression of the mRNAs of the two isoforms of Ccwap65 were different. The expression of Ccwap65-1 mRNA was not related to hypothermal stress in FW individuals. The expression of Ccwap65-1 mRNA was, however, down-regulated under hypothermal stress in SW individuals (Fig. 4a). On the other hand, under hypothermal stress, the expression of Ccwap65-2 mRNA was up-regulated in both the SW and FW groups (Fig. 4b).

In the acute phase, the expression of Ccwap65-1 mRNA was not significantly different upon the hypothermal challenge in the FW group (Fig. 5a). However, its expression in the SW individuals was down-regulated after 48 h of the hypothermal challenge (Fig. 5b). The expression of Ccwap65-2 mRNA was up-regulated after 96 h (Fig. 6a) and 12 h (Fig. 6b) upon the hypothermal challenge in the FW and SW individuals, respectively.

3.5. Differential patterns of Ccwap65 expression after administration of lipopolysaccharide (LPS)

The qPCR analyses showed that the LPS injection did not change the Ccwap65-1 mRNA levels in both FW- and SW-acclimated milkfish (Fig. 7a). On the other hand, it induced the expression of Ccwap65-2 mRNA after 24 h, with the expression being 2- and 4-folds higher than in the PBS-injected control groups in FW and SW, respectively (Fig. 7b).

3.6. Profiles of immune-response related genes in the milkfish transcriptome database

The expression profiles of the immune-response related genes under hypothermal stress were checked based on their FPKM (fragments per kilobase of transcript per million) values in the milkfish transcriptome database. Among them, interferon regulatory factor 3 and 7 (irf3 and irf7) were found to be up-regulated in FW- and SW-acclimated milkfish (Fig. 8). The members of the Toll-like receptor family, including tlr2, tlr3, tlr5, tlr11, and tlr22, were also identified in the milkfish database. Their expression showed the same pattern of up-regulation under the cold stress in both the FW and SW groups (Fig. 8). Moreover, the expression of tumor necrosis factor alpha (tnf) was observed to increase by 3- and 4-folds in the liver of FW and SW milkfish, respectively, under the cold stress. Based on the FPKM values, the downstream immune-response related genes, like gp130 and interleukin-6 receptor (il6r), were also found to be up-regulated after the cold challenge in both FW and SW milkfish.

3.7. Expression of immune-response related genes under hypothermal stress

Under hypothermal stress, the expression of four immune-response related genes were further analyzed by qPCR. The expression levels of liver irf3 mRNA in both the FW and SW groups were significantly affected by temperature (cold) (Fig. 9a; two-way ANOVA analysis, temperature factor: $F_{1,23} = 17.98$, $p < 0.001$;
**Fig. 2.** Phylogenetic analysis of the amino acid sequences of the Wap65 family. The phylogenetic tree was constructed taking into account the neighbor-joining method after bootstrapping (1000 bootstrap replications). The accession numbers of the different proteins are listed in the Supplementary Table 1.

**Fig. 3.** Expression of \textit{wap65-1} (a) and \textit{wap65-2} (b) in different tissues of milkfish in seawater, as detected by qPCR. Values are means ± SEM (n = 3 for all the groups). B, brain; G, gill; H, Heart; I, intestine; K, kidney; L, liver; M, muscle; S, spleen.
Fig. 4. Expression of hepatic *wap65-1* (a) and *wap65-2* (b) in the fresh water (FW) and seawater (SW) milkfish acclimated to 28°C (white bar) and 18°C (striped bar). Different letters (a and b) indicate significant differences between the 28°C and 18°C groups, and x and y indicate significant differences between the FW and SW groups as assessed by Student’s *t*-test pairwise comparison followed by two-way ANOVA. Values are means ± SEM, *n* = 6, *p* value < 0.05.

Fig. 5. Time-course of the expression of hepatic *Ccwap65-1* in the milkfish after hypothermal treatment (18°C) in (a) fresh water (FW) and (b) seawater (SW). Values were normalized with respect to the expression of *Ccgapdh2*. Values are means ± SEM, *n* = 6. The milkfish in the control group were sampled at 0 h and those in the hypothermal treatment group were sampled after 1, 3, 6, 12, 24, 48, 96, and 168 h post-cooling, respectively. The asterisks indicate significant differences as determined using one-way ANOVA (Dunnett’s comparison, *p* value < 0.05).
salinity factor: F_{1,23} = 0.02, p = 0.887; two factor interaction: F_{1,23} = 0.54, p = 0.470). The expression of irf7 mRNA showed the interaction between temperature (cold) and salinity responses.

Temperature (cold) effects on expression levels are significant in FW milkfish, but not in SW individuals. At 28°C, irf7 mRNA was more abundant in SW milkfish rather than FW ones (Fig. 9b; two-way ANOVA analysis, temperature factor: F_{1,23} = 17.66, p < 0.0001; salinity factor: F_{1,23} = 1.57, p = 0.225; two factor interaction: F_{1,23} = 22.64, p < 0.001). The il-6r (Fig. 9c) and gp130 (Fig. 9d) mRNA expression levels showed significant differences upon hypothermal treatment in livers of both FW and SW milkfish (Fig. 9c; two-way ANOVA analysis, temperature factor: F_{1,23} = 9.67, p = 0.006; salinity factor: F_{1,23} = 0.35, p = 0.559; two factor interaction: F_{1,23} = 1.75, p = 0.210; Fig. 9d; two-way ANOVA analysis, temperature factor: F_{1,23} = 20.61, p < 0.001; salinity factor: F_{1,23} = 0.06, p = 0.808; two factor interaction: F_{1,23} = 1.44, p = 0.244).

Fig. 6. Time-course of the expression of hepatic Ccwwap65-2 in the milkfish after hypothermal treatment (18°C) in (a) freshwater (FW) and (b) seawater (SW). Values were normalized with respect to the expression of Ccgapdh2. Values are means ± SEM, n = 6. The milkfish in the control group were sampled at 0 h and those in the hypothermal treatment group were sampled after 1, 3, 6, 12, 24, 48, 96, and 168 h post-cooling, respectively. The asterisks indicate significant differences as determined using one-way ANOVA (Dunnett’s comparison, p value < 0.05).

4. Discussion

In this study, two isoforms of warm-temperature-acclimation protein 65 kDa protein (wap65) gene were identified from the liver of milkfish. These two isoforms may play different roles in response to hypothermal stress. Kikuchi first identified Wap65 protein in the hepatopancreas of warm-temperature acclimated goldfish (Carassius auratus) [4]. Wap65 of goldfish was recognized to belong to the Wap65-1 subgroup from the phylogenetic tree analysis. In the past decade, studies on Wap65 isoforms in several teleosts revealed their responses to the challenges of temperature, pathogens, and heavy metals [5,7,10,25,26]. In addition, both Wap65 and hemopexin (Hpx) were found to function in iron homeostasis because of their affinity for the toxic, free heme, in blood circulation [3]. The CcWap65-1 and CcWap65-2 proteins have several highly conserved heme-binding pockets when compared to the human Hpx. CcWap65-1 contained five heme pockets in the
predicted amino acid sequence and CcWap65-2 contained only four pockets. In addition, the Ccwap65-2 sequence lacking the signal peptide is unique compared with other teleosts [5-7,10,15]. Although the signal peptide plays an important role in protein secretory pathway, non-classical protein secretion without an N-terminal signal peptide was found[27]. The analysis using the bioinformatics prediction tools (SecretomeP server) indicates that the CcWap65-2 may secret via the non-classical secretion pathway [27]. Moreover, the peptide sequence of CcWap65-2, unlike that of CcWap65-1, had higher identity and similarity with that of the mammalian Hpx. The CcWap65-2 sequence was observed to be more evolutionarily conserved than the CcWap65-1 sequence. Studies on other teleosts have also reported such kind of evolutionary differences [3,5].

In teleosts, wap65 has been identified as an immune-response gene having two isoforms. Kikuchi et al. first reported that in goldfish, wap65-2 expression was induced by bacterial LPS [28]. Sha et al. further demonstrated that in catfish the expression of wap65-2 was regulated by bacterial (Edwardsiella ictalrui) infections rather than by the warm-temperature stress [26]. Other studies also reported that the expression of Wap65-2 is an immune response in several teleosts, including ayu (Plecoglossus altivelis) [11,15], European seabass (Dicentrarchus labrax) [14], Japanese seabass (Lateolabrax japonicas) [29], mud loach (Misgurnus mizolepis) [7], olive flounder (Paralichthys olivaceus) [10], and rockbream (Oplegnathus fasciatus) [6]. In this study, we found that in milkfish, both the isoforms of Ccwap65 were mainly expressed in the liver, but the expression of only Ccwap65-2 was induced by the LPS injection.

Fig. 7. Effects of lipopolysaccharide (LPS) injection on Ccwap65-1 (a) and Ccwap65-2 (b) expression in fresh water (FW)- and seawater (SW)-acclimated milkfish. The milkfish were injected with 100 μL of phosphate-buffered saline (PBS; control) or 5 μg/g LPS in PBS (LPS). The liver was sampled 24 h after the injection. The expression levels were analyzed using qPCR. Values were normalized with respect to the expression of Ccgapdh2. Significance was analyzed using the Student’s t-test pairwise comparison followed by two-way ANOVA. Values are means ± SEM, n = 3, p value < 0.05.

Fig. 8. Differential expression of immune-response genes in fresh water (FW)- and seawater (SW) milkfish acclimated to 28 °C (white bar) and 18 °C (striped bar). The gene expression levels were evaluated by FPKM value in the milkfish transcriptome database [21]. Il6r: interleukin-6 receptor; IRF: interferon regulatory factor; TLR: toll-like receptor; TNFα: tumor necrosis factor-alpha.
Although wap65-2 was found to be more sensitive to pathogen infection than wap65-1 in most of the studied species, the wap65-2 expression in the liver of turbot (*Scophthalmus maximus*) was not induced by pathogen infection [5]. Under cold stress, the aquaculture gilthead sea bream (*S. aurata*) was reported to be infected with pathogens, like the *Pseudomonas* sp., *Aeromonas*, enterobacteria, and some viruses [18]. Different environmental salinities affect the cold-tolerant and antioxidant ability of milkfish [20,22,30]. Therefore, it is suggested that under hypothermal stress SW- and FW-acclimated milkfish have different response-patterns to various pathogen infections.

Hpx is an acute-phase response plasma protein released by mammalian livers, usually upon inflammation. With high scavenging ability of heme in the circulatory system, Hpx is an anti-inflammatory cytokines, IL-6 and TNF-α, in the bone marrow of mice [32]. In the teleostean fish, ayu (*P. altivelis*), the expression of wap65-2 in the liver was induced after 12 h of infection with *Listonella anguillarum*, and changes in the level of Wap65-2 were also detected in the serum after 4 h [11,15]. The wap65-2 transcripts in the liver of the Japanese sea bass (*L. japonicas*) were up-regulated after 24 h of infection with *Vibrio harveyi*. Immunoblot analysis of the Wap65-2 protein also showed significant increase in the serum of sea bass [29]. When infected with *Edwardsiella tarda*, acute responses in the expression of wap65-2 in liver were reported in flounder (*P. olivaceus*), mud loach (*M. mizolepis*), and rockbream (*O. fasciatus*) [6,7,10]. In this study, the expression of wap65-2 mRNA in milkfish was up-regulated after 24 h of LPS injection, but that of Ccwap65-1 was not changed. Therefore, hepatic Ccwap65-2 is an acute phase immune-response gene that was also up-regulated after 12 h and 96 h of hypothermal challenge in FW and SW, respectively. In FW milkfish, the energy resources may be used for other physiological responses in the acute phase. Being ectothermic, the immunity and resistibility of fish to pathogens can be affected by changes in the environmental temperature [33]. In addition, the resistibility of euryhaline milkfish to pathogens may be affected by the environmental salinities to enhance the differential immune responses under hypothermal stress.

As revealed from the milkfish transcriptome database [21], several immune-response genes were up-regulated under cold stress in FW- and SW-acclimated milkfish. Among them, toll-like receptors (TLRs), with highly conserved sequences as those in the mammals, were identified in teleosts. The pathogen-induced TLR signaling pathways triggered different TLRs and the consequent immune responses [34,35]. The interferon regulatory factors (IRFs),
including IRF3 and IRF7, function as the active Type I interferon genes and induce innate response. Moreover, upon inflammatory stimulus, the tumor necrosis factor alpha (TNF-α) was induced by LPS, other bacterial compounds, and interleukin-1 [35]. Furthermore, qPCR assessment revealed that the expression of irf3 mRNA was up-regulated in both FW and SW milkfish under cold stress, whereas that of irf7 was significantly increased in FW milkfish only. The expression of irf3 and irf7 were induced after 24 h of treatment with poly I:C and 3 h of treatment with LPS, respectively, in the large yellow croaker (Larimichthys crocea) [36]. In addition, treatment with poly(I:C) induced the expression of irf3 and irf7 mRNAs in the liver of the European eel (Anguilla anguilla). However, the expression of eel irf3 was higher compared to that of irf7 upon treatment with LPS [37]. Therefore, different patterns in the expression of irf3 and irf7 in milkfish under cold stress might be due to different pathogen infection. Moreover, increased expression of the down-stream immune-response genes, il6r and gpl30, for the regulation of immune responses and inflammation were reported in the liver of rainbow trout (Oncorhynchus mykiss) upon treatments with LPS and poly I:C [38], similar to the patterns found in livers of milkfish under cold stress. The patterns of up-stream and down-stream immune-response genes under cold stress thus further implicated that the status of milkfish were under pathogen infection.

In summary, two isoforms of Wap65 that are highly homologous to Hpx and have several highly conserved heme-binding domains were identified in the milkfish liver. The expression of hepatic Ccwap65-2 was significantly increased under hypothermal stress and after LPS injection. In the milkfish cultured under different salinity conditions, the expression of Ccwap65-2 showed an acute increase (12 h) in SW, whereas it was increased after 96 h (4 d) in FW milkfish. Our results indicated the pathogen infection status of milkfish under cold stress. Rapid increase of the Ccwap65-2 expression under hypothermal stress suggested a more efficient response to pathogen infection in SW milkfish compared with that in FW milkfish.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.09.012.

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