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# Effects of salinity on cold tolerance of Malaysian red tilapia

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**Abstract** This study was conducted to investigate the effect of salinity on growth, survival, osmoregulation, metabolism, hematological and biochemical parameters and T-complex protein (TCP-1) mRNA expression levels in Malaysian red tilapia at declining tank water temperatures. Five salinity concentrations (0, 5, 10, 15, 20 ‰) were evaluated during the study period. Samples were collected on a range of biochemical parameters at 16.5 (week 2), 12 (week 4) and 9.5 °C (week 6). No significant growth (body weight) was showed in the study ($P > 0.05$). Survival was monitored in the groups of fish until the holding tank temperature reached 5.6 °C. The lowest temperature in the study population of Malaysian red tilapia tolerated was 9 °C, although the fish were able to survive in temperature of 5.6 °C if they had been reared at 10 ‰ water salinity. The salinity and change in water temperature had significant effects on hematological and biochemical parameters and the TCP-1 gene expressions in Malaysian red tilapia ($P < 0.05$). The effect of temperature and salinity on superoxide dismutase (SOD), malondialdehyde (MDA) and hemoglobin (HGB) levels was significant ($P < 0.05$ or $P < 0.01$); with decreasing temperature, SOD and MDA activities increased, whereas HGB concentrations decreased. With increasing level of salinity, the SOD, MDA and blood glucose levels significantly increased ($P < 0.05$). The highest cholesterol (TC), triglycerides (TG), red blood cells (RBC) and HGB values were apparent in the moderate salinity groups, whereas the highest high-density lipoprotein cholesterol (HDL-C) was in the moderately high-salinity group. Rearing at low temperature, $Na^+$–$K^+$-ATPase (NKA) increased with increasing level of salinity at 16.5 °C, although it initially declined before rising again at water temperatures 12 and 9.5 °C. Rearing at low temperature, fish in the low-salinity groups significantly up-

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regulated the expression of the TCP-1 genes ($P < 0.05$), whereas fish in mild to moderate salinity groups significantly down-regulated the expression ($P < 0.05$). Taken together, these results suggest that exposure to environmental conditions of low temperature, low salinity and high salinity influence osmoregulation, metabolic parameters and the fish physiology. However, a change in the isotonic point at cold temperatures was observed by rearing in a salinity of under 10 ‰. This could potentially provide a means to increase cold tolerance of overwintering Malaysian red tilapia.

**Keywords** Red tilapia · Temperature · Salinity · Cold tolerance · T-complex protein (TCP-1)

## Introduction

Tilapia is a globally important species in aquaculture because of its rapid growth rate, firm and flavorsome flesh and ease of reproduction in captivity. Furthermore, strong resistance to environmental stresses makes them well suited for aquaculture (Thodesen et al. 2011). Since their introduction to the region, a large-scale farming of tilapia has formed in southern China. Fishes of the family Cichlidae, to which tilapias belong, are abundant in tropical and subtropical regions. However, the inability of tilapia to tolerate low temperatures is of major economic concern as it reduces their growing season and leads to few survival over winter, leading to enormous economic losses (Charo-Karisa et al. 2005; Kocher 2008).

Temperature is one of the most influential factors affecting the growth, survival, physiological function, biochemical functions and immune function of fish (Moreira et al. 2007; Qiang et al. 2014). At low temperatures, three changes occur in the fish. Firstly, cold temperature activates the neuroendocrine pathway causing the release of cortisol, thyroid hormone or catecholamines (Chen et al. 2002; Chebaani et al. 2014); Next, changes of osmoregulatory, hematological, blood enzymatic and metabolic function occur (Chebaani et al. 2014; Younis 2015); Finally, abnormal behavior, growth inhibition, increased susceptibility to infection and deaths of fish are observed (Ndong et al. 2007; Ma et al. 2015). The lower lethal temperature varies due to environmental effects, genetic effects and gene–environment interaction at the individual fish level as a result of exposure history, and so estimating the lowest tolerable temperature for tilapia is difficult (Cnaani et al. 2000).

Although essentially a freshwater fish, many tilapia species are euryhaline and so can be cultured in fresh, brackish or salt water (Kang'Ombe and Brown 2008; Hassan et al. 2013). Previous studies have demonstrated that salinity may significantly affect the growth, osmoregulatory, metabolic modifications, physiological and biochemical parameters and the heat shock protein (Hsp) gene expressions of some species (Tine et al. 2010; Vargas-Chacoff et al. 2011; Velan et al. 2011; Laverty and Skadhauge 2012; Baysoy et al. 2013; Vargas-Chacoff et al. 2015). The ability to accurately quantify osmolality and coordinate a response of appropriate magnitude over a range of stress levels suggests that tilapias may have evolved novel osmoregulatory mechanisms (Wang et al. 2009). Salinity can also affect metabolic costs and growth rates of both adults and juveniles, even in species capable of surviving under a range of salinity levels (Lehtonen et al. 2016). Further, it has been shown that tilapia can avoid dehydration and adapt to the hyperosmolality of seawater (Jiang et al. 2008). The studies of Al-Amoudi (1987) in *Oreochromis aureus*, *Oreochromis niloticus* and *Oreochromis mossambicus* and Kamal and Mair (2005) in the *O. niloticus*

and *O. mossambicus* revealed that species of tilapia vary in salinity tolerance and that optimal salinity is between 3 and 18 ‰. Many researchers previously proved that fish in isotonic medium has good physiological conditions (Peterson et al. 2005; Kang'Ombe and Brown 2008; Hassan et al. 2013; Vargas-Chacoff et al. 2015). Hassan et al. (2013) even indicated that isotonicity of the environment may enhance the cold tolerance of *O. niloticus*; and Kang'Ombe and Brown (2008) studied the effect of salinity on growth, feed utilization and survival of juvenile tilapia *rendalli* in tanks, and the results indicated that salinity of 10 ‰ is optimal for T. *rendalli* in tank culture. However, some studies suggested that there was a limited influence (Stauffer 1986; Jennings 1991).

In recent years, red tilapia has been increasingly used for aquaculture production in many parts of the world, such as China, Malaysian and Thailand (Jayaprasad et al. 2011). While the genetics of red tilapia varieties are not well documented, their derivation is generally attributed to the crossbreeding between mutant reddish-orange *Mozambique tilapia* (*O. mossambicus*) with other tilapia species like *Nile tilapia* (*O. niloticus*) and blue tilapia (*O. aureus*). Red tilapia has gained popularity due to its very fast growth rate, absence of black membrane in the body cavity, salinity tolerance and adaptability to most culture systems (Pradeep et al. 2014). However, in the main red tilapia production area in south of China, red tilapia cultured in soil pond and lack of heating equipment in winter, which always caused lots of death when there was a sudden cooling. Acclimation to low salinity (10–15 ‰) reduces the lower water temperature limit of many tilapiine fishes seems to be a good method (Zale and Gregory 1989).

There is, however, a lack of research on the interaction between salinity and temperature in Malaysian red tilapia. The present experiment was carried out to investigate the effect of temperature, salinity and their interaction on survival, osmoregulatory, metabolism, hematological and biochemical parameters and T-complex protein (TCP-1) genes expressions in Malaysian red tilapia under laboratory conditions and to identify a potential optimal rearing salinity to help increase cold tolerance of Malaysian red tilapia. The aim was to provide theoretical and experimental basis to support the breeding of cold tolerant Malaysian red tilapia, and a method to improve cold tolerant that could be overwintered without or declined production losses.

## Materials and methods

### Fish and experimental design

Malaysian red tilapia sample fish were provided by the Freshwater Fisheries Research Center (FFRC), Chinese Academy of Fishery Sciences (Wuxi, China). Fish with a mean body weight of 43.10 ± 3.90 g and mean standard length of 102.15 ± 5.14 mm were cultured in an indoor recirculation aquaculture systems (Zhongkehai Recirculation Aquaculture Systems Co., Ltd., Qingdao, China) for a week. Each plastic tank (500 L) with 250 L treated water (that had been aerated for consecutive 3 days) was equipped with a heating device to maintain the water temperature at 25 °C, and fish were fed twice per day with commercial tilapia floating pellets (Tongwei Co. Ltd., Wuxi, China).

Four-hundred and fifty red Malaysian tilapias were randomly selected from the holding tank and divided into five groups (each group has three parallel sets) at a density of 30 fish/500-L plastic tank (water depth: 50 cm, volume: 250 L). Individual groups were placed one to each of the following salinities: concentration of 0, 5, 10, 15 and 20 ‰, labeled $S_0$,

$S_5$, $S_{10}$, $S_{15}$ and $S_{20}$, respectively. Salinity was increased by the addition of synthetic sea salt (Lanhaixing Co. Ltd., Hangzhou, China) at a rate of 5 ‰ every second day starting with the $S_{20}$ tank and following the order outlined in Table 1 to ensure that all tanks reached target salinity at the same time (Table 1). After this was reached, the regulated tank temperature was decreased by 1 °C every day by regulating the heating device until it reached the natural water temperature (2 days, from 25 to 23 °C) and then removed the heating device to simulate natural environmental conditions.

After that, the experiment began and lasted for 50 days. Water exchange was carried out every 3 days, pH = 7.5–7.9, dissolved oxygen (DO) > 9 mg L-1 and $NH_4$-N < 0.5 mg L-1. Aeration was supplied to each tank 24 h per day, and photoperiod was 12D:12 L.

## Sampling and measurements

Fish were sampled at week 2 (water temperature 16.5 °C), week 4 (water temperature 12 °C) and week 6 (water temperature 9.5 °C). Final body weight of samples was recorded. Blood, liver and gill samples were collected from three randomly selected fish from each set. Fish were bled from the tail vein using a 2.5-mL syringe pretreated with anticoagulant based on sodium citrate (4 %). Blood samples were immediately divided into two parts and transferred to sterile tubes. The first part was separated by centrifugation (3500 rpm for 10 min) to isolate the plasma. The plasma was used to measure the total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and blood glucose (GLU) levels by automatic biochemical analyzer (Roche Modular P800, Germany). The second part of blood sample was mixed with anticoagulant based on sodium citrate (4 %) and then used to detect blood cell contents by automatic blood cell analyzer (Mindray, BC-2800, Shenzhen, China).

The liver and gill samples were immediately placed frozen at −20 °C for later analysis of liver superoxide dismutase (SOD), liver malondialdehyde (MDA) and gill $Na^+$–$K^+$-ATPase (NKA) activities. These indicators were undertaken with kits manufactured by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Partial liver tissues were collected and frozen at −80 °C for later analysis of gene expression as described below.

## Analysis of TCP-1 mRNA expressions

Total RNA was isolated using the Kit (DR104) of Yuanpinghao (Biotech Co. Ltd., Tianjin, China) following the manufacturer's instructions. The concentration and quality of RNA were, respectively, determined by BioPhotometer plus (OD260: OD280) (Eppendorf, Germany) and agarose gel electrophoresis (AGE). Then, 500 ng cDNA synthesis was performed using total RNA with oligo-dT primers and reverse transcription system (Takara Bio Inc., Dalian, China). PCR amplification was carried out with cDNA on Applied

**Table 1** Protocol for adjusting holding tank salinity

| Group | Day 1 | Day 3 | Day 5 | Day 7 |
|---|---|---|---|---|
| $S_0$ | 0 | 0 | 0 | 0 |
| $S_5$ | 0 | 0 | 0 | 5 |
| $S_{10}$ | 0 | 0 | 5 | 10 |
| $S_{15}$ | 0 | 5 | 10 | 15 |
| $S_{20}$ | 5 | 10 | 15 | 20 |

Synthetic sea salt was added at a rate of 5 ‰ every 2 days beginning with $S_{20}$ on Day 1

Biosystems 7500 Fast RT-PCR System (Applied Biosystems, USA) under the following conditions: Stage 1: initial denaturation at 95 °C for 30 s, 1 cycle; Stage 2: 40 cycles of 95 °C for 15 s and 60 °C for 1 min. β-Actin was employed as a housekeeping gene, and each sample was analyzed in triplicate. Amplification reaction was conducted by the Applied Biosystems 7500 Fast RT-PCR System.

To establish the PCR reaction system, a total volume of 20 μL was made up of 10 μL SYBR Premix Ex Taq (2×) (Takara, Dalian); 2 μL template; 0.8 μL forward primer (10 μmol/L); 0.8 μL reverse primer (10 μmol/L); 0.4 μL ROX Reference Dye II (50×) *2; and 6 μL double-distilled water. The RT-PCR primers of TCP-1-eta, TCP-1-beta and β-actin gene (Table 2) were designed against the known tilapia cDNA sequence. The comparative CT method ($2^{-\Delta\Delta CT}$) was used to assess the relative level of genes normalized to the reference gene (β-actin), and the results were compared with the control group (S0, 16.5 °C) with one-way ANOVA analysis using SPSS 17.0.

### Statistical analysis

The results were expressed as mean ± standard error. Data of physiological and biochemical indicators were analyzed by two-way ANOVA, and the significant differences which were compared by Duncan's multiple comparisons. Data of TCP-1 genes expression levels were compared by one-way ANOVA. The data were analyzed by SPSS software (version 17; SPSS Inc., Chicago, USA) and considered very significant at a level of $P < 0.01$ and significant at a level of $P < 0.05$.

## Results

### Effect of salinity on survival rate of Malaysian red tilapia exposed to declining water temperature

The results of the survival analysis (shown in Fig. 1) revealed that fish in the highest salinity tanks, $S_{20}$, were the first to reach 50 % survival rate (CS = 50 %), followed by $S_{15}$ and the low-salinity group, $S_0$, with the moderate salinity groups, $S_5$ and $S_{10}$, exhibiting the best survival rate. Fish in group $S_0$ maintained a higher survival rate while in 25–13 °C range, whereas the survival rate declined rapidly once below 13 °C. The $S_{20}$ group was the first to reach zero survival, achieved at a temperature of 9.5 °C. This was followed by the $S_{15}$ and S0 groups at 9 °C, and the $S_5$ and $S_{10}$ groups were the last at 6.3 and 5.6 °C, respectively.

**Table 2** Sequences of primers used in the study

| Primer | Sequence (5′-3′) | NCBI GenBank accession no. |
|---|---|---|
| β-Actin F | GTACCACCATGTACCCTGGC | FN673689.1 |
| β-Actin R | TGAAGTTGTTGGGCGTTTGG | |
| TCP-1-eta F | GATGGCTGCCAGGTGATTGC | JQ797421.1 |
| TCP-1-eta R | CACCAGAGTTTTGGCCGCAG | |
| TCP-1-beta F | CGCCGTCATGAGGCTGAAAG | JQ797420.1 |
| TCP-1-beta R | ATCAGTGTCCATGCCGGTGT | |

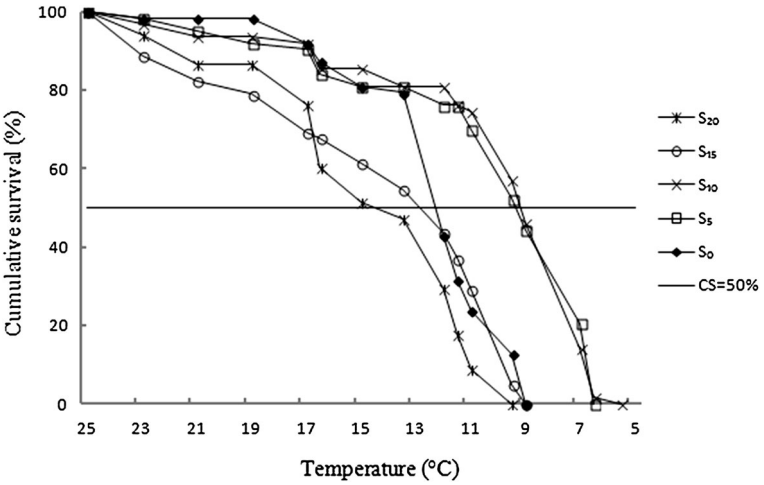


**Fig. 1** Cumulative survival rate (%) at declining holding temperatures in different salinity groups. CS = 50 % means 50 % survival. *Data* represent the mean ± SE ($P < 0.05$)

The semi-lethal temperature (LT50) in $S_0$ and $S_{15}$ groups was between 13 and 11 °C, that of the $S_5$ and $S_{10}$ groups was between 9 and 7 °C and $S_{20}$ group was between 15 and 13 °C.

## Effect of salinity on NKA activity of Malaysian red tilapia exposed to declining water temperature

The results of NKA activity in Malaysian red tilapia are presented in Fig. 2. At 16.5 °C, NKA activities in $S_{15}$ and $S_{20}$ groups were significantly higher than in the $S_0$ group ($P < 0.05$). At 12 °C, no difference was observed in NKA activity among all groups

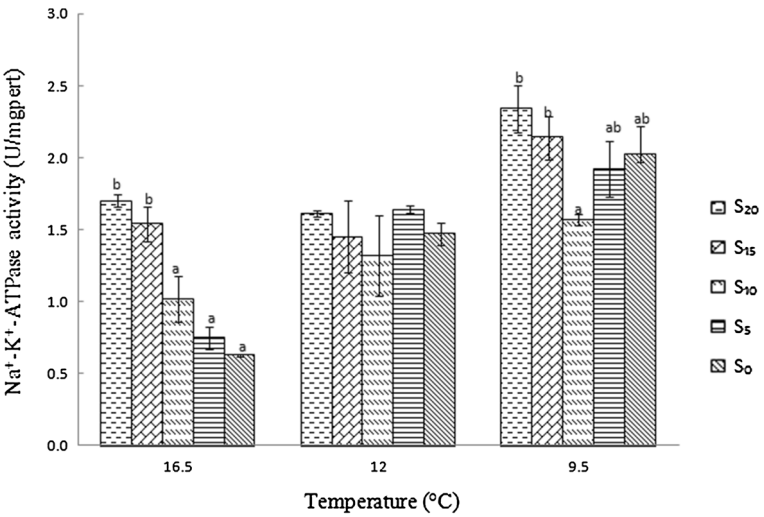


**Fig. 2** $Na^+$–$K^+$-ATPase (NKA) activity in Malaysian red tilapia maintained in different salinities and temperatures. *Statistical significance* is represented by *different letters*, compared to the $S_0$ group at different temperatures ($P < 0.05$, $n = 9$)

($P > 0.05$). At 9.5 °C, NKA activities in $S_{15}$ and $S_{20}$ groups were significantly higher than in the $S_0$ group ($P < 0.05$).

### Effect of salinity on growth, biochemical markers in Malaysian red tilapia exposed to declining water temperature

As given in Table 3, two-way ANOVA revealed that both temperature and salinity significantly affected SOD, MDA, TC, TG, HDL-C, GLU, RBC and HGB levels ($P < 0.05$ or $P < 0.01$). The interaction between temperature and salinity had a significant effect on SOD and MDA activities and HGB level ($P < 0.05$ or $P < 0.01$) but no significant effect on TC, TG, HDL-C, GLU and RBC levels ($P > 0.05$). However, among temperature and salinity, as well as the interaction between temperature and salinity had no significant effect on body weight of red tilapia ($P > 0.05$). At the same salinity and with decreasing temperature, the antioxidant indicators of SOD and MDA activities gradually increased, while HGB level decreased. Similarly, as salinity increased, SOD and MDA activities significantly increased ($P < 0.05$). Indicators related to body metabolism such as TC, TG and HDL-C levels increased at first and then decreased. The TC and TG levels in the $S_{10}$ group were significantly higher than the control group ($S_0$) ($P < 0.05$). The HDL-C level in the $S_{15}$ group and the GLU level in $S_{20}$ were significant higher than the control group ($S_0$) ($P < 0.05$). The RBC level gradually decreased as salinity increased, and in the $S_{15}$ and $S_{20}$ groups were significantly lower than the control group ($S_0$) ($P < 0.05$).

### Effect of salinity on TCP-1 mRNA expression in Malaysian red tilapia exposed to declining water temperature

The results showed that the initial values of TCP-1-eta and TCP-1-beta expressions in $S_5$, $S_{10}$ and $S_{15}$ groups was significant higher than the control group ($S_0$, 16.5 °C) ($P < 0.05$) and significantly decreased with the temperature decline ($P < 0.05$) (Figs. 3, 4), while the initial values of TCP-1-eta and TCP-1-beta expressions in $S_{20}$ group had no significant difference with the control group ($S_0$, 16.5 °C) ($P > 0.05$), and the expressions of TCP-1-eta and TCP-1-beta genes in $S_0$ and $S_{20}$ groups significantly increased with the temperature decreases ($P < 0.05$).

## Discussion

To the best of the authors' knowledge, this is the first time that the interactive effect of temperature and salinity on survival, physiological and the expressions of cold-resistant genes have been studied for Malaysian red tilapia overwintering. Water temperature was a predominant factor influencing survival of fish. Our results indicated that drops in temperature could cause direct survival of red tilapia during overwintering. Decreased temperatures during overwintering have been shown to reduce feeding events of red tilapia (data are not showed in this paper), potentially making them less energy to resist the cold. Furthermore, cold temperatures could induce bacteria disease to lead to few survival of red tilapia (Martins et al. 2011). Our results found no significant survival were observed after a few days of exposure to brackish, which confirmed the high plasticity of red tilapia. However, a lower of survival was occurred at high salinity than the low salinity with decrease in water temperature, suggested that the fish body was overloaded to handle the

**Table 3** Effect of salinity on growth, hematological and biochemical parameters of Malaysian red tilapia exposed to declining water temperatures

| Temperature | Salinity | Parameters | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Final body weight (g) | SOD (nmol/mL) | MDA (U/mL) | TC (mmol/L) | TG (mmol/L) | HDL-C (mmol/L) | GLU (mmol/L) | RBC ($10^{12}$/L) | HGB (g/L) |
| 16.5 °C (week 2) | $S_0$ | 43.38 ± 1.26 | 74.50 ± 4.98 | 7.95 ± 0.37$^{a}$ | 2.24 ± 0.07$^{a}$ | 1.75 ± 0.03$^{a}$ | 1.02 ± 0.10$^{a}$ | 21.21 ± 1.46$^{a}$ | 0.44 ± 0.021$^{b}$ | 11.22 ± 0.22$^{ab}$ |
| | $S_5$ | 43.66 ± 1.09 | 85.95 ± 1.67 | 6.05 ± 0.38$^{a}$ | 2.85 ± 0.08$^{a}$ | 1.88 ± 0.09$^{ab}$ | 1.11 ± 0.01$^{a}$ | 20.90 ± 0.19$^{a}$ | 0.44 ± 0.017$^{b}$ | 12.67 ± 0.51$^{b}$ |
| | $S_{10}$ | 43.58 ± 1.40 | 85.51 ± 2.89 | 6.16 ± 0.63$^{a}$ | 3.31 ± 0.10$^{b}$ | 2.41 ± 0.15$^{b}$ | 1.28 ± 0.10$^{ab}$ | 18.84 ± 0.20$^{a}$ | 0.51 ± 0.020$^{b}$ | 12.56 ± 0.48$^{b}$ |
| | $S_{15}$ | 43.64 ± 1.11 | 74.94 ± 3.94 | 7.68 ± 0.70$^{a}$ | 2.83 ± 0.28$^{ab}$ | 2.05 ± 0.22$^{a}$ | 1.36 ± 0.07$^{b}$ | 20.23 ± 0.73$^{a}$ | 0.34 ± 0.018$^{a}$ | 8.67 ± 0.51$^{a}$ |
| | $S_{20}$ | 42.20 ± 1.40 | 76.45 ± 5.07 | 11.69 ± 0.87$^{b}$ | 2.41 ± 0.14$^{a}$ | 1.69 ± 0.21$^{a}$ | 1.28 ± 0.03$^{ab}$ | 24.74 ± 2.55$^{b}$ | 0.37 ± 0.023$^{a}$ | 9.56 ± 1.22$^{a}$ |
| 12.0 °C (week 4) | $S_0$ | 42.37 ± 3.40 | 94.71 ± 10.48$^{a}$ | 5.45 ± 0.28$^{a}$ | 1.83 ± 0.06$^{a}$ | 1.33 ± 0.07$^{a}$ | 0.85 ± 0.07$^{a}$ | 20.29 ± 0.56$^{a}$ | 0.38 ± 0.037b | 9.56 ± 0.29$^{ab}$ |
| | $S_5$ | 43.98 ± 1.39 | 94.61 ± 3.55$^{a}$ | 5.57 ± 0.08$^{a}$ | 1.79 ± 0.08$^{a}$ | 1.64 ± 0.16$^{ab}$ | 0.93 ± 0.07$^{a}$ | 30.25 ± 1.01$^{a}$ | 0.33 ± 0.016b | 7.33 ± 0.44$^{a}$ |
| | $S_{10}$ | 43.75 ± 0.78 | 134.65 ± 3.12$^{b}$ | 7.17 ± 0.26$^{a}$ | 3.00 ± 0.05$^{b}$ | 1.97 ± 0.11$^{b}$ | 0.97 ± 0.10$^{ab}$ | 24.72 ± 0.60$^{a}$ | 0.34 ± 0.025b | 11.67 ± 1.20$^{b}$ |
| | $S_{15}$ | 41.40 ± 1.76 | 154.18 ± 4.07$^{c}$ | 10.60 ± 0.45$^{b}$ | 2.67 ± 0.24$^{ab}$ | 1.48 ± 0.10$^{a}$ | 1.21 ± 0.07$^{b}$ | 30.05 ± 1.69$^{a}$ | 0.24 ± 0.012a | 7.39 ± 0.96$^{a}$ |
| | $S_{20}$ | 41.96 ± 1.37 | 162.70 ± 0.95$^{c}$ | 11.19 ± 1.40$^{b}$ | 1.61 ± 0.08$^{a}$ | 1.30 ± 0.24$^{a}$ | 1.15 ± 0.10$^{ab}$ | 45.80 ± 3.51$^{b}$ | 0.21 ± 0.007a | 7.17 ± 0.25$^{a}$ |
| 9.5 °C (week 6) | $S_0$ | 42.10 ± 1.25 | 145.55 ± 1.59$^{a}$ | 8.20 ± 0.38$^{a}$ | 1.36 ± 0.13$^{a}$ | 0.64 ± 0.02$^{a}$ | 0.84 ± 0.06$^{a}$ | 36.65 ± 1.23$^{a}$ | 0.29 ± 0.013$^{b}$ | 7.25 ± 1.25$^{b}$ |
| | $S_5$ | 43.28 ± 1.21 | 151.82 ± 2.59$^{a}$ | 7.57 ± 0.26$^{a}$ | 1.93 ± 0.17$^{a}$ | 1.53 ± 0.15$^{ab}$ | 0.97 ± 0.05$^{a}$ | 34.44 ± 1.17$^{a}$ | 0.24 ± 0.006$^{b}$ | 5.89 ± 0.11$^{ab}$ |
| | $S_{10}$ | 42.12 ± 1.62 | 153.79 ± 2.72$^{ab}$ | 7.79 ± 1.13$^{a}$ | 2.30 ± 0.19$^{b}$ | 1.70 ± 0.22$^{b}$ | 0.97 ± 0.09$^{ab}$ | 31.40 ± 1.45$^{a}$ | 0.32 ± 0.042$^{b}$ | 7.11 ± 0.70$^{b}$ |
| | $S_{15}$ | 40.85 ± 1.81 | 162.83 ± 4.05$^{b}$ | 9.94 ± 0.31$^{b}$ | 1.92 ± 0.20$^{b}$ | 1.18 ± 0.08$^{a}$ | 1.08 ± 0.02$^{b}$ | 37.81 ± 1.93$^{a}$ | 0.22 ± 0.000$^{a}$ | 5.75 ± 0.25$^{ab}$ |
| | $S_{20}$ | 40.72 ± 1.59 | 168.14 ± 8.70$^{b}$ | 11.41 ± 0.28$^{b}$ | 1.29 ± 0.14$^{a}$ | 0.96 ± 0.10$^{a}$ | 0.97 ± 0.08$^{ab}$ | 48.39 ± 1.79$^{b}$ | 0.16 ± 0.015$^{a}$ | 3.50 ± 0.50$^{a}$ |
| Two-way ANOVA | | | | | | | | | | |
| Temperature | | NS | ** | * | ** | ** | ** | ** | ** | ** |
| Salinity | | NS | ** | ** | ** | ** | ** | ** | ** | ** |
| Temperature × salinity | | NS | ** | * | NS | NS | NS | NS | NS | * |

Different superscript letter in the same column signify statistical differences ($P < 0.05$, n = 9; mean ± SE)

* Significant at the 5 % level ($P < 0.05$); ** Significant at the 1 % level ($P < 0.01$); *NS* not significant ($P > 0.05$)

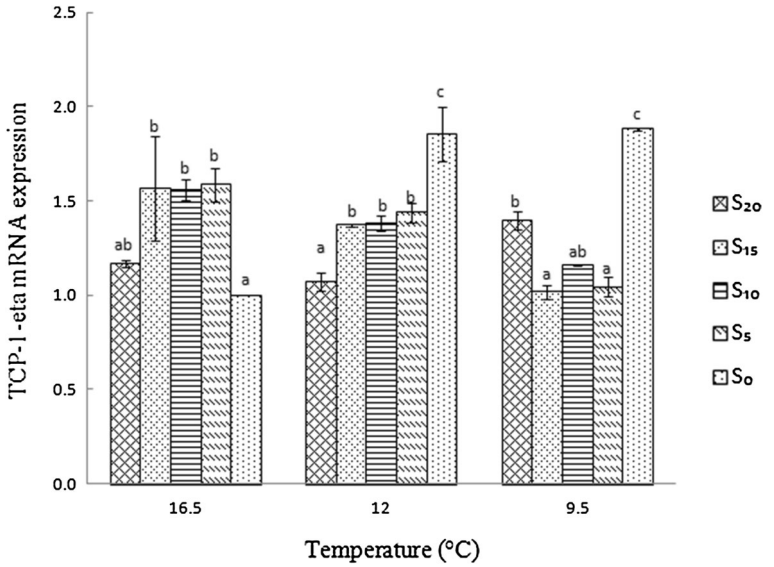


**Fig. 3** T-complex protein-1-eta (TCP-1-eta) mRNA expression in liver of Malaysian red tilapia. *Statistical significance* is represented by *different letters* compared to $S_0$ group at different temperatures ($P < 0.05$, $n = 3$)

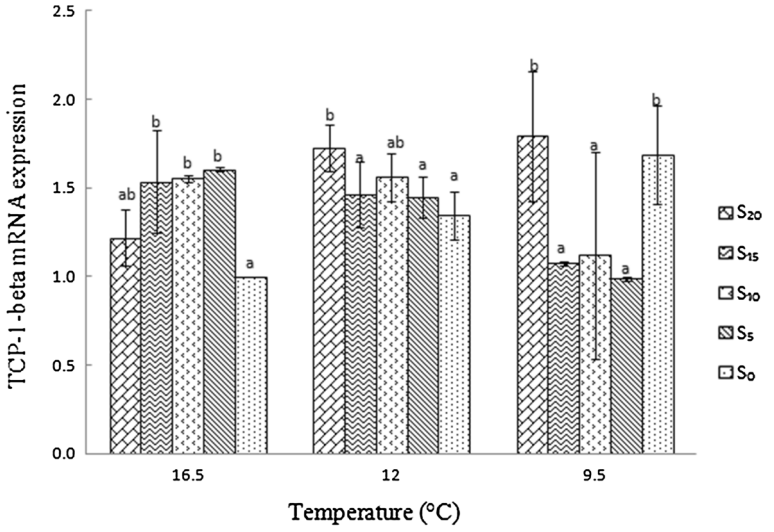


**Fig. 4** T-complex protein-1-beta (TCP-1-beta) mRNA expression in liver of Malaysian red tilapia. *Statistical significance* is represented by *different letters* compared to $S_0$ group at different temperatures ($P < 0.05$, $n = 3$)

cold temperature and high salinity at the same time; or, although described as euryhaline, red tilapia could not live for long periods of time in high-salinity water habitats. The inability of red tilapia to develop long-term hypo-osmoregulation in freshwater probably limits the upstream penetration of this species. Our results agreed with those reported for

other species, where environmental salinity close to the isosmotic point (between 10 and 15 ‰) induced the high survival during cold temperature (Kang'Ombe and Brown 2008; Hassan et al. 2013).

The lowest-temperature fish can tolerate is hard to estimate, for the lower lethal temperature was influenced by many factors, such as the body mass, the species, environmental effects, history of the fish and the genetic bases of cold tolerance (Cnaani et al. 2000; Charo-Karisa et al. 2005; Kang'Ombe and Brown 2008; Saillant et al. 2008). A lowest lethal temperature of 5.6 °C was found in $S_{10}$ group, and the semi-lethal temperature from 9 to 7 °C was observed in both $S_5$ and $S_{10}$ groups. However, Li et al. (2002) reported that the Egyptian strain of *O. niloticus* cultured in China had the best cold tolerance for the first mortality at 11 °C and total mortality at 7.4 °C; Charo-Karisa et al. (2005) investigated the cold tolerance of juvenile *Nile tilapia* and found that fish mortality started at 13.6 °C and total mortality occurred at 8.6 °C, and smaller fish (<5 g) were more susceptible to lower temperature than larger fish; and Zhu et al. (2013) reported that the lowest tolerant temperature of Malaysian red tilapia was 6.4 °C and the semi-lethal temperature was 7.6 °C. This discrepancy may be attributable to the difference in time exposure to water temperature and salinity stress. Acute reductions in water temperature may influence fish survival directly via limits on physiological tolerance or indirectly by acting as a sublethal stressor (VanDeHey et al. 2013). Lermen et al. (2004) proved that the chronic thermal exposures resulted in greater metabolic changes than acute exposure. The lowest lethal temperature that was observed in $S_{10}$ may be related to less energy spent on osmoregulatory strategies as well as better utilization of energy, investing it in physiological processes or resisting the cold. Zale and Gregory (1989) reported that irrespective of whether above or below the isotonic salinity, the cold tolerance of blue tilapia (*O. aureus*) was reduced. Sardella and Brauner (2007) also reported that high salinity could cause *Mozambique tilapia* osmoregulatory failure in cold temperature and eventually lead to mass mortality in winter. Peterson et al. (2005) reported that juvenile blue tilapias *O. aureus* had an isosmotic media at 11.6 ‰, where survived at lower temperatures than those in water of higher or lower salinity.

Growth is the manifestation of the net outcome of energy gains and losses within a framework of abiotic and biotic conditions (Kumlu et al. 2000). The present study showed that no significant growth (body weight) in red tilapia was observed throughout the experiment. It may be due to under the conditions of cold temperature and salinity, much energy was used to enhance metabolism for resisting the cold and osmoregulation, lead to less energy to growth.

Osmoregulation is essential to fish life and is energetically expensive (Hassan et al. 2013). Osmoregulatory balance was achieved through efficient regulation of the $Na^+$, $K^+$-ATPase (NKA) activity in different osmoregulatory tissues (gill, kidney and intestine), and consequently fish was able to ionoregulate and maintain its plasma osmolality constant throughout the salinity range tested(Laverty and Skadhauge 2012; Vargas-Chacoff et al. 2015). The gill epithelium was in direct contact with water and thus represents a major site for osmotic water loss and diffusive gain of salts. According to the changes of NKA activity at 16.5 °C, which exhibits a direct and positive relationship with respect to water salinity, and correlates with the change in direction and magnitude of net salt transport by the gill following seawater adaptation at the early stage (Laverty and Skadhauge 2012). Similar observations of increased gill NKA activity have since been made on numerous species (Arjona et al. 2009; Vargas-Chacoff et al. 2011, 2014). Obviously, NKA activity also had strong positive relationship with osmolality (Herrera et al. 2009; Tomy et al. 2009; Vargas-Chacoff et al. 2014).

According to the changes of NKA activity at 12 and 9.5 °C after a long time (more than 14 days) regulation and adaption to the salinity environment, a hyposmotic environment of 5 ‰ and hyperosmotic environment of 15 and 20 ‰ both forced the gills to increase their energy expenditure in osmoregulation, and thus, the NKA activities in hyposmotic and hyperosmotic environment were higher than that in isotonic environment (around 10 ‰). Similar results of NKA activity at 12 and 9.5 °C were recorded by Vargas-Chacoff et al. (2015) for *E. maclovinus* juveniles (gill) exposed to 5–31 ‰ environmental salinities for 90 days, showed a ''U-shaped'' relationship to water salinity, which suggested that more than 14 days are required for tilapia to reach the chronic regulatory period, where osmoregulatory and metabolic parameters reach a new homeostatic point(Vargas-Chacoff et al. 2015); Similar results also reported by Hassan et al. (2013) for tilapia, Vargas-Chacoff et al. (2014) for *Eleginops maclovinus* and Kang et al. (2015) for milkfish (*Chanos chanos*). Hassan et al. (2013) reported that tilapias from fresh water into isotonic medium water, in parallel, the water temperature drop from 25 to 14 °C for 168 h, suggested that the least disturbance of ionic balance caused by cold tolerance is occurred in the isotonic medium, and the energy saved from osmoregulation may have contributed to cold tolerance energy requirements.

In order to survive in the cold water and to maintain the stability of internal environment under cold stress and salinity stress, fish need to regulate osmotic pressure. From the osmoregulation mechanism we had explained above, we known that red tilapia had highly energy consumption at hypotonic and hypertonic environment to maintain their internal ionic and osmotic balance, and less energy was likely required to maintain ion balance in an isosmotic environment, where the ionic gradient between extracellular fluid and water is minimal (Kang'Ombe and Brown 2008).

According to the changes of plasma glucose (GLU), the salinity environment of 10 and 20 ‰ had the minimum and maximum GLU. And all salinity groups increased with decreasing levels of temperature, our findings are in agreement with the findings of grouper (*Epinephelus malabaricus*), who reported that the grouper (*E. malabaricus*) increased serum cortisol and glucose within 30 min to stabilize the plasma osmolality after transferred to high salinity exhibited physiological changes; a possible explanation for the changes of GLU was that the glucose metabolism enzyme activity of fish intensified at hypotonic and hypertonic environment, as well as the cold temperature, causing a mass mobilization of liver glycogen, then lead to higher plasma GLU. Meanwhile, The metabolic balance in low salinity (5 ‰) and high salinity (15 and 20 ‰) acclimated fish showed increased hepatic gluconeogenesis, demonstrated by the decrease in TC, TG and HDL-C, as another way to obtain energy for high energy expenditure in osmoregulation at those salinities. Associated with the increase in plasma GLU and adipose concentrations at hyposmotic and hyperosmotic environment, as well as low temperature, suggesting that demands protein and adipose (triacylglycerol, cholesterol and high-density lipoprotein) as energy sources in addition to sugars.

Alternatively, under prolonged cold stimulation, the glucose metabolism system may instead have become dysregulated with many enzymatic reactions unable to be completed within effective time, and accordingly, the pathway of glucose metabolism to ATP would be blocked (Inoue et al. 2008). This could explain why blood glucose level was not reduced in the current study and inconsistent with other studies (Martínez-Álvarez et al. 2002; Arjona et al. 2009). The results highlight the low energetic costs of isotonic medium for red tilapia, which suggests efficient osmoregulation capacities.

Low temperature exposure not only can lead to collapse in cellular structure and cell division but also likely to result in enhanced metabolism with increased oxygen

consumption to meet higher demands for energy. The increased oxygen consumption in turn is likely to result in enhanced production of reactive oxygen species (ROS), which could cause substantial harm to the cells (Paital and Chainy 2010; Yu et al. 2015). In order to remove excess ROS, MDA and SOD activities increased. Our results are, generally, in accordance with the previous statement, where oxidative stress is highly sensitive to temperature, due to temperature-induced ROS production (Vinagre et al. 2012; Madeira et al. 2013) and dependent on the magnitude of the thermal stress, suggested that cell response against cold stress includes also increases the activities of SOD and CAT, which are key enzymes for directly scavenging reactive oxygen species. Moreover, according to the changes of SOD and MDA activities in different salinity environments, hyperosmotic environment combination with cold temperature was also considered harmful for the fish (Fonseca et al. 2011) for much energy consumption. However, when the antioxidant defenses are inadequate to combat the action of the ROS, the result is oxidative stress (Martínez-Álvarez et al. 2002). Changes in the levels of blood parameters give an insight into the health status of an individual (Harikrishnan 2011), which was increasingly used as indicators of the physiological stress response to endogenous or exogenous changes in fish (Lermen et al. 2004). According to the changes of RBC and HGB, S10 group was always the significantly higher than that of other salinity groups at varying temperature. Our findings are in agreement with the findings of Shahkar et al. (2015), who reported that Juvenile Ship Sturgeon (*Acipenser nudiventris*) had higher RBC and HGB at 8 ‰ group than the low- (0 and 4 ‰) and high-salinity (12 ‰) groups, suggesting that the change in environmental salinity can be attributed to changes in the water content in the blood. At the beginning of exposure to a hyperosmotic environment, the fish would lose water passively, and thereby undergoing increases in the concentrations of blood cell elements. Afterward, the compensatory increase in water ingestion would provide a transitory dilution of the blood parameters. Finally, these would return to initial values as a result of the rest of the osmoregulatory mechanisms, which act to re-establish the extracellular volume (Martínez-Álvarez et al. 2002). Interestingly, the current study noted that fish feeding reduced with decreasing temperature, and thus, nutrient intake of material needed for RBC and HGB. As a result, RBC and HGB levels were reduced. It was reported that the movement of fish was reduced in lower temperature, leading to reduce of oxygen consumption (Ibarz et al. 2003). Therefore, as a carrier to transport $O_2$ and $CO_2$, respectively, RBC and HGB levels were correspondingly reduced.

Chaperonins containing TCP-1 (CCT) belong to heat shock proteins 60 family (Hsp60), which can help other proteins in assembling, folding and translocating, and play a role in protecting cells against injuries and other types of stress (Yu et al. 2015). Hsps appear to be widely used by organisms subjected to cold environments (Rinehart et al. 2007); And as a molecular chaperone that could assist in protein refolding under stress, the CCT complex is believed to be a key component contributing to cellular homeostasis under adverse condition (Shimon et al. 2008). On this study, a further experiment was conducted to investigate whether salinity might interact with low temperatures to affect the expression of TCP-1 genes in red tilapia.

Rinehart et al. (2007) discovered a member of the Hsp60 family, TCP-1, is up-regulated during flesh fly diapauses, showed that showing the up-regulation of a microtubule-specific chaperonin during diapause further highlights the importance of cytoskeletal stability during diapause and low-temperature survival, proposing that up-regulation of Hsps during diapause is a major factor contributing to cold hardiness of overwintering insects. Gracey et al. (2004) reported that carp were exposed to increasing level of cold, and responses assessed by using a microarray showed that the expressions of TCP-1-epsilon and

TCP-1-eta were up-regulated, reported that when the wine yeast (*Saccharomyces cerevisiae*) were exposed to temperature as low as 4 °C, the expressions of CCTS α and CCTS β increased by between threefold and fourfold, the authors attributed this up-regulation to the depolymerization of tubulins and actins. According to the previous research, both actin and tubulin are believed to engender depolymerization at low temperature when cytoskeleton is subjected to crumbling, which is a significant element for the occurrence of cold injuries.

According to the changes of TCP-1 gene expressions, expressions of TCP-1-eta and TCP-1 beta genes up-regulated in $S_0$ and $S_{20}$ and down-regulated in $S_5$, $S_{10}$ and $S_{15}$, suggesting that TCP-1-eta of red tilapia contributes significantly to the winter cold tolerance; moreover, salinity have shown a profound effect in increasing cold tolerance of red tilapia. Particularly, the salinity around the isosmotic environment is optimal for Malaysian red tilapia to defense the cold stress. Similar observations of increased liver TCP-1 genes have since been made on other species, Yu et al. (2015) reported that relative mRNA transcript levels of SpCCTα in muscle of subadult *S. paramamosain* following exposure to low temperatures (10, 15, 20 and 25 °C) in combination with both high (35) and low salinity (10), showed that 10°C group most significantly higher levels than those of 25 and 20 °C, indicated that the SpCCTα gene was connected with the cold hardiness of *S. paramamosain*; moreover, SpCCTα gene of 10 °C–10 ‰ group was up-regulated at the early 6 h and then down-regulated until the end of experiment (48 h), and 10 °C–35 ‰ group had the similar trend that up-regulated at the early 12 h and then down-regulated, proved that salinity could interact with the temperature to modify the impacts of temperature on aquatic organisms.

## Conclusion

In the present study, the influence of salinity on growth, survival, osmoregulation, metabolism, biochemical and expressions of cold resistance genes of Malaysian red tilapia was assessed. Our results provide some empirical confirmation that the lowest temperature for Malaysian red tilapia tolerance in freshwater is 9 °C, although the survival temperature can be as low as 5.6 °C when the fish is maintained at 10 ‰ of salt solution. No growth of red tilapia is showed in this study. Osmoregulatory processes cause major physiological changes in the fish. The results indicated that acclimation of red tilapia to isosmotic conditions (around 10 ‰) enhanced the survival overwintering and resistance to cold water. Malaysian red tilapia presents a better cold tolerance in a range that goes from their isosmotic point (10 ‰). Energy expenditure in osmoregulation is minimal at 10 ‰, while increased at both low and high salinity due to the activity of gills. We documented for the first time significant changes in TCP-1 gene expressions in Malaysian red tilapia reared at different temperatures and different salinities and provide evidence that numerous Hsps are developmentally up-regulated during the overwintering of the Malaysian red tilapia. We thus suspect that the functions of salinity during overwinter contribute to the enhancement of cold tolerance not only by promoting the fluidity between inside and outside the cell membrane, but also by decreasing in energy consumption and oxidation.

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