Zoological Studies
a SpringerOpen Journal

RESEARCH ARTICLE Open Access

Short-term effects of thermal stress on the responses of branchial protein quality control and osmoregulation in a reef-associated fish, *Chromis viridis*

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Abstract

Background: Changes in ambient temperature seriously affect physiological regulation and biochemical reactions in ectotherms. However, transient elevation in oceanic temperature occurs naturally during the day. Short-term elevation in the ambient temperature affects different physiological responses in marine fish, including cellular protein stability and osmotic balance of the internal environment. Since fish gills are vital osmoregulatory organ which directly contacts external environment, activation of cytoprotective responses to maintain gill cell viability and biological function is essential for fish survival under challenging environmental conditions. The purpose of this study was to investigate the short-term effects of elevated temperature on physiological regulation in the gills of a marine teleost, blue green damselfish (*Chromis viridis*).

Results: As part of the stress response, plasma glucose levels were induced by short-term hyperthermic exposure (12 h). Furthermore, upregulation of the levels of gill heat shock proteins (HSPs) and ubiquitinated proteins was essential for preventing the accumulation of protein aggregations in branchial cells of *C. viridis* under hyperthermic stress. The specific activity of branchial Na^+/K^+-ATPase (NKA), however, significantly reduced while the amount of protein was similar between normal and high-temperature groups.

Conclusions: The present study provided the evidence to illustrate that activation of the branchial protein quality control mechanism to carry out cytoprotective response was involved in coping with thermal stress. However, plasma osmolality and muscle water content, respectively, that slightly but evidently increased and decreased might result from impaired osmoregulatory ability due to hyperthermia-decreased gill NKA activity.

Keywords: Thermal stress; Blue-green damselfish; Protein quality control; Osmoregulation

Background

Most organisms on Earth are ectotherms which have to survive and adapt to temperature fluctuations (Hochachka and Somero 2002; Guschina and Harwood 2006; Somero 2010). Temperature fundamentally affects all aspects of physiology by influencing the reactive rates as well as the physical properties of biological molecules (Hochachka and Somero 2002; Crockett and Londraville 2006). For marine ectotherms including fish, environmental temperature has the pervasive effects on physiological and biochemical functions at all levels of biological organization, from molecule to organism (Jobling 1995; Hochachka and Somero 2002; Hofmann et al. 2002; Donaldson et al. 2008). According to the tolerance range of temperature, the fishes can be classified into two groups, eurythermal and stenothermal species. Eurythermal fish can maintain metabolic activity at temperatures as low as Antarctic fish can survive and withstand temperature as high as the avian and mammalian body temperature. In contrast, changes in environmental temperature may lead to the poor maintenance of physiological homeostasis in stenothermal

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species, resulting in temperature stress (Hochachka and Somero 2002; Somero 2010; Long et al. 2012).

Under adverse conditions, the physiological stress responses of organisms should be promptly activated to correct the disturbance, and cytoprotective mechanisms should be employed for the maintenance of cell viability and functional activity; otherwise, the survival of stressed organism will be in danger (Hofmann and Somero 1995, 1996; Hofmann et al. 2002; Kregel 2002; Cui et al. 2013). Furthermore, cellular proteins indeed carry out various physiological functions responsible for cell viability. The expression and maintenance of protein quality depends on mechanisms beyond those involved in transcription and translation (Wickner et al. 1999). Chaperones and proteases mediating the mechanism of protein quality control (PQC) to prevent the accumulation of aggregated proteins and maintain cellular function and activity are highly conserved in organisms from different taxa (Gottesman et al. 1997; Wickner et al. 1999; Goldberg 2003; Bukau et al. 2006). Therefore, PQC should be a critical cytoprotective mechanism for coping with temperature stress in ectotherms. However, study on the responses of PQC mechanism to temperature challenge in fish is limited. The fish gills are the multifunctional organ which directly contacts the external environment (Evans et al. 2005; Kaneko et al. 2008); therefore, it is an excellent model to study stress responses and environmental effects in vivo.

Environmental temperature significantly influences internal electrolyte and osmotic homeostasis in aquatic ectotherms (Christensen 1975; Amoudi et al. 1996; Metz et al. 2003; Sardella et al. 2004, 2008a). It is due to active ion-transporting mechanisms that are regulated by many proteins, while the cellular proteins of stenothermal species are only marginally stable at a limited range of temperature (Hochachka and Somero 2002; Metz et al. 2003; Crockett and Londraville 2006; Sardella et al. 2008a). The fundamental transporter proteins responsible for osmoregulation in gill epithelia have been reported in previous studies (see Hirose et al. 2003; Evans et al. 2005; Hwang and Lee 2007; Kaneko et al. 2008; Hwang et al. 2011). Among them, Na^+/K^+-ATPase (NKA) is the most important enzyme that actively transports Na^+ out of and K^+ into animal cells for sustaining intracellular homeostasis as well as for providing the driving force for ion-transporting systems in fish gills (Hwang and Lee 2007; Hwang et al. 2011). Therefore, branchial NKA responses (mRNA and protein expression and specific activity) have been used to assess the osmoregulatory status/ability of teleosts (Epstein et al. 1967; Hwang and Lee 2007; Kaneko et al. 2008). In this regard, it is worth examining the branchial NKA responses to investigate the impact of temperature stress on osmoregulatory responses in stenothermal teleosts and clarify whether the protein expression or specific activity of gill NKA is susceptible to temperature challenge.

The blue-green damselfish (*Chromis viridis*) is a stenothermal teleost that is abundant on coral reefs throughout much of the Indo-Pacific region (Allen 1991; Lieske and Myers 1994), including southern Taiwan (Shen et al. 1993). Previous studies have shown that the average temperature in Nanwan Bay, Kenting National Park, southern Taiwan is 26°C to 27°C and increases to approximately 32°C to 33°C during the day (Meng et al. 2008; Mayfield et al. 2013). Accordingly, the normal and hyperthermic temperatures of 26°C and 32°C, respectively, were used in this study. The goal of this study was to investigate the stress responses, PQC mechanism, and osmoregulatory response in the gills of *C. viridis* exposed to an increase in ambient temperature (32°C) for 12 h to ascertain the physiological strategies employed by stenothermal teleosts under short-term thermal stress.

Methods

Experimental animals and environments

Blue-green damselfish 2.8 ± 0.4 g in weight and 4.1 ± 0.7 cm in length were obtained from husbandry center of National Museum of Marine Biology and Aquarium (NMMBA), Pingtung, Taiwan. Fish were reared in a tank with a 300 L seawater (SW, 33% to 35‰) circulating system at 26 ± 0.5°C with a daily 12-h photoperiod at least 4 weeks for the holding period. The waters were continuously circulated through fabric-floss filters, and the environmental salinity was measured by the refractomter PAL-06S (ATAGO, Tokyo, Japan). Fish were fed daily with commercial pellets (TetraMarin®, Tetra, Melle, Germany) except 48 h prior to the sampling. No mortality was observed during the holding period. For all following experiments, 28 individuals were sacrificed. The facilities and protocols for the experimental fish were approved by the Institutional Animal Care and Use Committee of College of Marine Sciences, Nation Dong Hwa University (i.e., NMMBA).

Short-term exposure of blue-green damselfish to elevated temperature

After the holding period, blue-green damselfish were randomly divided into two different groups for the control and hyperthermic treatment. The temperature was maintained at 26°C ± 0.5°C for control group and 32°C ± 0.5°C for hyperthermic group. A 100 W automatic heater (EBO-JÄGER, El Segundo, CA, USA) was used to maintain the temperature. After 12-h short-term exposure, the experimental animals were randomly selected from two tanks and anesthetized by immersion in MS-222 (50 mg/l) before sampling.

Analysis of plasma glucose levels, plasma osmolality, and muscle water content

Fish blood was collected from the heart using heparinized 1 ml syringes and 21 G needles. After centrifugation

at 1,000 × *g* at 4°C for 10 min, the plasma osmolality and glucose levels were measured immediately using a Wescor 5520 Vapro osmometer (Logan, Utah, USA) and an ACCU-CHEK Go blood glucose meter (Roche, Mannheim, Germany), respectively. The muscle water content (MWC) was measured gravimetrically after drying at 100°C for 48 h. The procedures of analysis of plasma glucose levels, plasma osmolality, and MWC were determined according to Tang and Lee (2013b).

Antibodies

The primary antibodies used in the present study included (1) anti-heat shock protein 90 (HSP90) (1:1,500 dilution), a rabbit polyclonal antibody (#4874; Cell Signaling Technology, Beverly, MA, USA) corresponding to human HSP90; (2) anti-HSP70 (1:2500 dilution), a mouse monoclonal antibody (H 5147; Sigma, St. Louis, MO, USA) generated by immunization with purified bovine brain HSP70; (3) anti-HSP60 (1:1,000 dilution), a mouse monoclonal antibody (H3524; Sigma) recognizes an epitope located between amino acid residues 383–419 of the human; (4) anti-ubiquitin (1:2,000 dilution), a rabbit polyclonal antibody (#3933; Cell Signaling Technology) corresponding to the N-terminus of the human ubiquitin protein that detects ubiquitin, polyubiquitin, and ubiquitinated proteins; (5) anti-β-actin (1:5,000 dilution), a monoclonal antibody (ab8226, Abcam, Cambridge, England, UK) against residues 1–100 of human β-actin; and (6) anti-NKA (1:4,000 dilution), a mouse monoclonal antibody (α5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) raised against the α-subunit of avian NKA. The secondary antibodies for Western blot analyses were horseradish peroxidase (HRP)-conjugated goat anti-rabbit or anti-mouse IgG (Chemicon, Temecula, CA, USA). A 1:12,000 dilution of secondary antibodies was used in the present study.

Cell protein fractionation and isolation of aggregated proteins

The procedures of cell protein fractionation and isolation of aggregated proteins were performed according to published studies (Aufricht et al. 1998; Chen et al. 2002; Rinehart et al. 2006; Tang and Lee 2013a). The studied tissues were homogenized in chilled extraction buffer containing 0.1% Triton X-100, 60 mM PIPES, 1 mM EDTA, 1 mM ethylene glyco-bis(aminoethyl ether)-N,N,N,N-tetraacetic acid and 100 mM NaCl. In addition, 40 μl of a proteinase inhibitor cocktail (Roche, Mannheim, Germany) was added for each milliliter of chilled extraction buffer. Homogenization was performed in 2 ml tubes with a Polytron PT1200E (Lucerne, Switzerland) at appropriate speed for 10 s. The homogenate was centrifuged at 680 × *g* for 10 min at 4°C to pellet nuclei and large cellular fragments. The supernatant was assigned to the total cell lysates for the following analyses of HSPs and ubiquitinated proteins. The resulting supernatant (total cell lysate) was centrifuged at 35,000 × *g* for 14 min at 4°C to separate the Triton-soluble and insoluble protein fractions. Aggregated proteins were isolated by differential centrifugation. The Triton-insoluble fraction was resuspended twice in extraction buffer, sonicated, and pelleted at 17,000 × *g* for 30 min at 4°C. The resultant pellet was again resuspended in extraction buffer, sonicated, and pelleted at 5,000 × *g* for 30 min at 4°C. The pellet consisting of aggregated proteins was resuspended in extraction buffer (aggregated protein fraction) and stored at −80°C. Protein concentrations of total cell lysates and aggregated protein fractions were determined with a BCA Protein Assay Kit (Pierce, Hercules, CA, USA) using bovine serum albumin (BSA, Pierce) as a standard.

Preparation of crude membrane fractions

The procedure of preparation of crude gill membrane fractions was performed according to Tang et al. (2012). The gills of the fish were excised and blotted dry immediately after the fish were killed by spinal pithing. The samples were immersed in liquid nitrogen and placed into ice-cold homogenization buffer (250 mM sucrose, 1 mM EDTA, 30 mM Tris, pH 7.4). Homogenization was performed in 2 ml tubes using the Polytron PT1200E homogenizer (Lucerne, Switzerland) at appropriate speed for 10 s. Debris, nuclei, and lysosomes were removed by low-speed centrifugation (12,000 × *g* for 10 min, 4°C). The remaining supernatant was centrifuged at medium speed (20,800 × *g* for 1 h, 4°C). The resulting pellet was resuspended in homogenization buffer and stored at −80°C. The pelleted fraction contained large fragments of the plasma membrane along with membranes from the Golgi and the endoplasmic reticulum, but no small cytoplasmic vesicles as they typically do not pellet down unless greater forces (100,000 × *g* for >1 h) are applied (Alberts et al. 1994). This fraction is therefore referred to as the crude membrane fraction. Aliquots of crude cell membrane fractions were saved for protein determination analysis. Protein concentrations were determined with BCA Protein Assay Kit (Pierce) using bovine serum albumin (Pierce) as a standard. The crude membrane fractions were stored at −80°C until the analysis of Western blot and specific activity of gill NKA.

Western blot analysis

Gill proteins were heated in sample buffer at 90°C for 10 min for detection of HSPs in total cell lysates or at 37°C for 30 min for detection of NKA in crude membrane fractions. The samples were separated by electrophoresis on sodium dodecyl sulfate (SDS) containing 8% polyacrylamide gels for detection of HSPs and NKA. The prestained protein molecular weight marker was purchased from Fermentas (SM0671; Hanover, MD, USA). The separated proteins were

Table 1 Effects of short-term exposure to high temperature on physiological parameters of blue-green damselfish

Environments	NT	HT
Plasma glucose levels (mg/dL)	54.0 ± 2.6	100.2 ± 8.2*
Plasma osmolality (mOsm/kg)	347.1 ± 4.9	379.2 ± 6.7*
Muscle water content (%)	87.4 ± 0.8	79.5 ± 0.6*

Values are expressed as the mean ± SEM, $n = 6$ for all groups. NT, normal temperature (26°C ± 0.5°C); HT, high temperature (32°C ± 0.5°C). The asterisk indicated a significant difference ($P < 0.05$) by unpaired t test.

then transferred to PVDF membranes (0.45 μm pore size) (Millipore, Bedford, MA, USA) by electroblotting. After pre-incubation for 3 h in phosphate-buffered saline (PBS) (137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) with 0.075% (*v*/*v*) Tween 20, pH 7.4 (PBST) buffer containing 5% (*w*/*v*) nonfat dried milk to minimize nonspecific binding, the blots were incubated at room temperature for 3 h with primary antibody diluted in 1% BSA and 0.05% sodium azide in PBST, washed in PBST, and incubated at room temperature for 2 h with secondary antibody. The immunoreactive bands were developed with HRP substrate, Western Blot Enhancer Kit (T-Pro, New Taipei City, Taiwan), and imaged with a Fusion FX7 system (Vilbert Lourmat, Eberhardzell, Germany). β-actin was used as the loading control for HSPs. To verify even the loading of the crude membrane fractions, the protein amount of each lane on the blots was quantified after staining the membranes with Ponceau S (Romero-Calvo et al. 2010). The developed blots were imported as TIFF files. Immunoreactions were analyzed using a software package (MCID software, Imaging Research, Ontario, Canada). The results were converted to numerical values to compare the relative protein abundance of the immunoreactions.

Dot blot analysis

Levels of ubiquitinated proteins in the gills were measured using an immunochemical analysis modified from the study of Todgham et al. (2007). Equal amounts of total protein (10 μg) from each sample were blotted onto pre-wetted nitrocellulose membrane (0.2 μm pore size) (Sartorius, Epsom, Surrey, UK) in triplicates by gravity filtration using a BioDot dot blotter (Bio-Rad, Hercules, CA, USA). Wells were washed twice with 200 μl of PBST and then heat-fixed at 65°C for 20 min. Then, the membrane was blocked in 5% (*w*/*v*) nonfat dried milk in PBST for 1.5 h. Following blocking, the membranes were washed three times in PBST (for 5 min each). The membranes were incubated at room temperature for 3 h with primary antibody (anti-ubiquitin antibody, Cell Signaling Technology) diluted in 1% BSA and 0.05% sodium azide in PBST, washed in PBST, and subsequently incubated at room temperature for 2 h with secondary antibody. The immunoreaction was developed with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and imaged with a Fusion FX7 system (Vilbert Lourmat, Eberhardzell, Germany). The developed membranes were imported as TIFF files. The immunoreactive signals were analyzed using a software package (MCID software). The results were converted to numerical values to compare the levels of ubiquitinated proteins of the immunoreactive signals.

Specific Na^+/K^+-ATPase activity

A method using 96-well microplate to measure the inorganic phosphate concentrations for determination of NKA activity was performed according to Tang et al. (2010) with minor modification. Aliquots of the suspension of gill crude membrane fractions, prepared as described above, were used to determine the protein concentration and NKA enzyme activity. The reaction medium (final concentration, 100 mM imidazole-HCl buffer, pH 7.6, 125 mM NaCl, 75 mM KCl, 7.5 mM $MgCl_2$) was prepared according to Tang et al. (2010). Then, 10 μl crude membrane fractions, 50 μl 10 mM ouabain (specific inhibitor of NKA) or deionized water, and 100 μl 10 mM Na_2ATP were added to 340 μl of the reaction medium. The enzyme activity was defined as the difference between the inorganic phosphate liberated in the presence and absence of ouabain in the reaction mixture. The reaction mixture was incubated at the exposure temperatures for 20 min followed by immediate ice bath for 10 min to stop the reaction (Cheng et al. 1999). Because the previous studies have demonstrated that the specific NKA activity which was measured at the exposed temperature of fish would correlate with the level of in vivo activity (Metz et al. 2003; Sardella et al., 2008a), therefore, the reaction was run at the exposure temperatures in this study. The concentration of inorganic phosphate was measured according to Doulgerakia et al. (2002). The colorimetric reagent consisted of 1% Tween-20 and 0.75% ammonium molybdate in 0.9 M H_2SO_4. The reaction mixtures and colorimetric reagent were mixed in a 1:1 (*v*/*v*) ratio, and then, the concentration of inorganic phosphate in each samples was determined by a microplate reader (VERSAmax, Molecular Devices, Sunnyvale, CA, USA) at 405 nm. Each sample was determined in triplicates. Some protocols determine the concentration of inorganic phosphate by measuring the color of molybdenum blue, which is the reduced product of phosphomolybdate. The instability of color formation and reagents, however, were variables in those protocols. The formation of the unreduced phosphomolybdate in the present study is directly proportional to the amounts of inorganic phosphate.

Statistical analysis

In all experiments, statistical significance was determined using Student's t test ($P < 0.05$) for group data analysis. Values were expressed as means ± S.E.M.

Results

Physiological parameters: plasma glucose, osmolality, and muscle water content

Compared to the normal temperature (NT, 26°C) group, the levels of plasma glucose of blue-green damselfish were significantly elevated after exposure to high temperature (HT, 32°C) condition. In addition, plasma osmolality and MWC which significantly increased and decreased, respectively, were found when blue-green damselfish were exposed to elevated temperature (Table 1).

Changes of the abundance of gill heat shock proteins and ubiquitin-conjugated proteins

In the cellular stress responses, the relative protein abundance of stress proteins (i.e., HSPs) in fish gills was examined. Immunoblotting of the gills (Figure 1) from NT- and

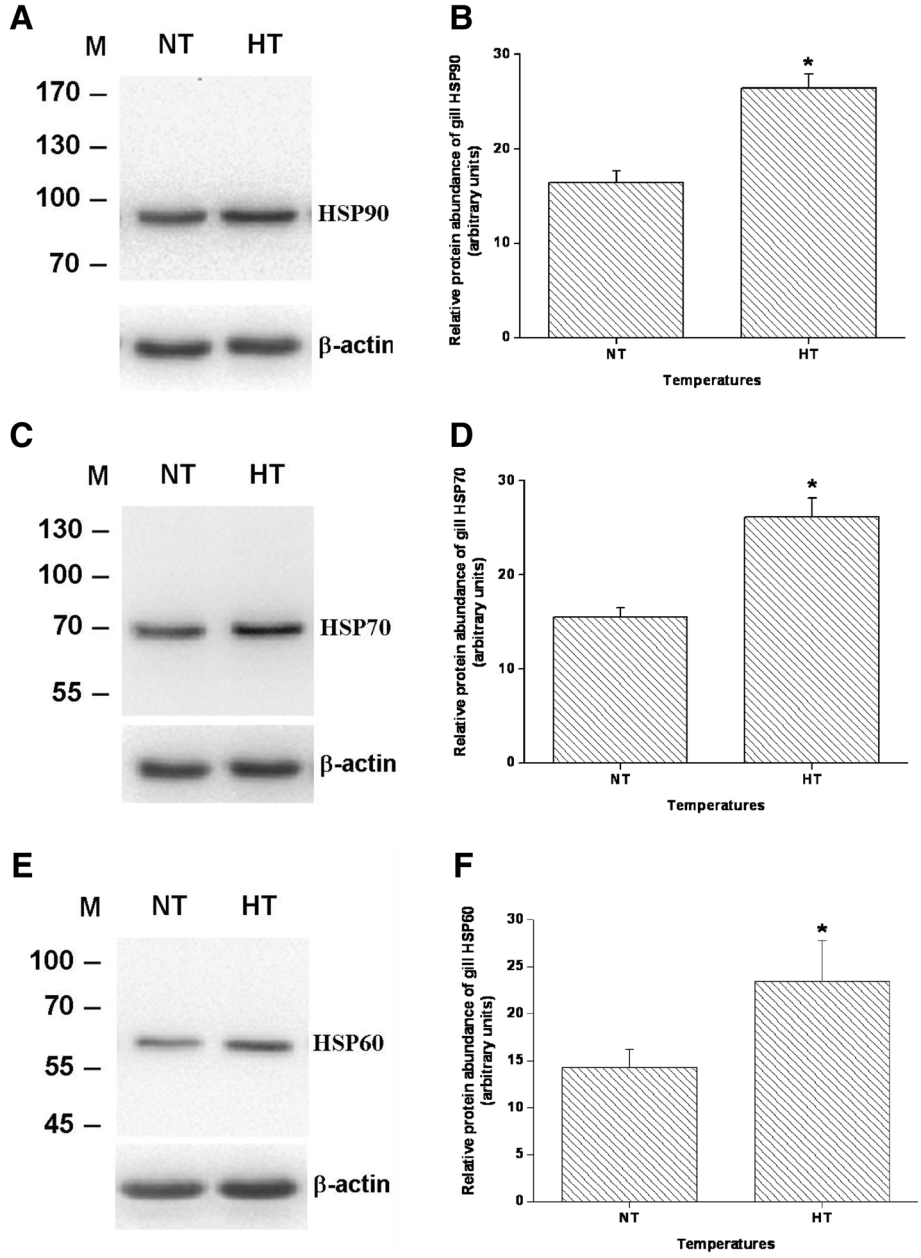

Figure 1 Protein expression of branchial heat shock protein 90 (HSP90), HSP70, and HSP60 in blue-green damselfish (*Chromis viridis*). The representative immunoblots of HSP90, HSP70, and HSP60 showed a single immunoreactive band with a molecular mass about 90 **(A)**, 70 **(C)**, and 60 kDa **(E)**, respectively. The immunoreactive bands of HT group are more intensive than that in the NT group. The protein amounts of HSP90 **(B)**, HSP70 **(D)**, and HSP60 **(F)** increased evidently after hyperthermic exposure ($n = 5$). β-actin was used as the loading control. The asterisk indicated a significant difference ($P < 0.05$) by unpaired t test. Values were mean ± SEM. M, marker; NT, normal temperature (26°C); HT, high temperature (32°C).

HT-exposed blue-green damselfish probed with primary antibodies to HSP90 (Figure 1A,B), HSP70 (Figure 1C,D), and HSP60 (Figure 1E,F) resulted in single immunoreactive bands with molecular weights of approximately 90 (Figure 1A), 70 (Figure 1C), and 60 kDa (Figure 1E), respectively. Protein expression levels of branchial HSPs in HT-exposed fish were significantly higher than those in NT-exposed fish (1.61-fold for HSP90, Figure 1B; 1.69-fold for HSP70, Figure 1D; and 1.64-fold for HSP60, Figure 1F). The response of ubiquitin-conjugated proteins in blue-green damselfish gills to reduced salinity was assayed by using dot-blot analysis. Dot-blot analysis showed the levels of ubiquitin-conjugated proteins in the gills of HT-exposed *C. viridis* were higher than those in NT-exposed *C. viridis* (Figure 2). Importantly, the aggregated proteins were maintained at similar levels between HT- (48.4 ± 10.5 μg/mg total protein) and NT-exposed fish (40.1 ± 7.8 μg/mg total protein) (Figure 3).

Na^+/K^+-ATPase responses

The relative protein abundance of branchial NKA was examined. Immunoblotting of the gills from NT- and HT-exposed blue-green damselfish obtained a single immunoreactive band with molecular weight of approximately 105 kDa (Figure 4A). The protein abundance of gill NKA α-subunit in *C. viridis* was similar between two studied environmental temperatures (Figure 4B). However, reduction of gill NKA specific activities was found in fish exposed to HT condition (Figure 4C).

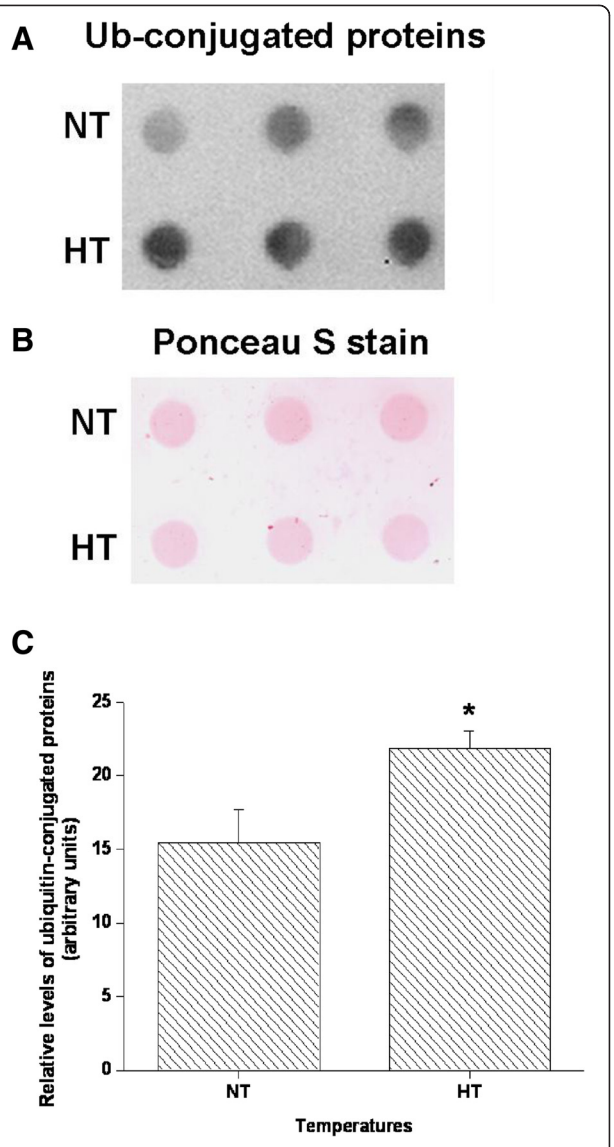

Figure 2 Dot-blot analysis of the levels of ubiquitin-conjugated proteins in the gills of blue-green damselfish (*Chromis viridis*). (A) Ubiquitin-conjugated protein levels were shown as relative values based on dot intensities. **(B)** Ponceau S total protein stain of blots was used as loading control. **(C)** Significant elevation of the levels of ubiquitin-conjugated proteins was found after hyperthermic exposure. Unpaired *t* test was used for the statistics. Values are means ± S.E.M ($n = 5$). NT, normal temperature (26°C); HT, high temperature (32°C).

Discussion

Organisms naturally experience diverse environmental challenges throughout their lives. For marine ectothermic organisms, ambient temperature is one of the most significant factors that affects diverse regulation (Hochachka and Somero 2002; Hofmann et al. 2002; Crockett and Londraville 2006; Donaldson et al. 2008; Somero 2010). In fish physiological responses, the mechanisms associated with stress and osmoregulatory responses are susceptible to variation of environmental temperature (Hofmann and Somero 1995; Iwama et al. 1999; Gonzalez and McDonald 2000; Metz et al. 2003; Place et al. 2004; Sardella et al. 2004; Fiess et al. 2007; Sardella et al. 2008a,b; Cui et al. 2011; Deane and Woo 2011; Feidantsis et al. 2012; Cui et al. 2013). For ecological relevance, the experimental temperatures used in this study were based on the average (26°C) and daytime (32°C) temperatures in Nanwan Bay, southern Taiwan (Meng et al. 2008; Mayfield et al. 2013) because *C. viridis* is abundant in Nanwan Bay (Shen et al. 1993).

The stress responses are energy demanding processes, changes in plasma glucose concentrations have widely been used as a stress bioindicator at the organismal level, because glucose is the main fuel source in animals (Basu et al. 2001; Afonso et al. 2003; Iwama et al. 2006). To evaluate whether a short-term increase in environmental temperature would thermally stress *C. viridis*, its plasma glucose concentrations were measured. Plasma glucose levels increased from 54.0 ± 2.6 to 100.2 ± 8.2 mg/dL after hyperthermic exposure (Table 1). Similar patterns were found in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) (Mesa et al. 2002) and two Antarctic nototheniid fish, *Pagothenia borchgrevinki* and *Trematomus bernacchii*

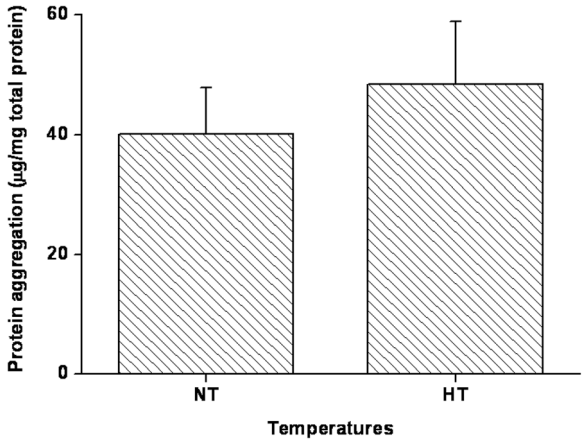

Figure 3 The levels of aggregated proteins in the gills of blue-green damselfish (*Chromis viridis*) exposed to different temperature. There was no significant difference found in the levels of aggregated proteins between NT and HT groups. Values are means ± S.E.M ($n = 5$). NT, normal temperature (26°C); HT, high temperature (32°C).

(Lowe and Davison 2005), after short-term exposure to thermal stress. Therefore, 32°C should be a stressful temperature for *C. viridis* and more energy is needed to compensate for the cost of the energy-demanding processes involved in coping with thermal stress.

At the cellular level, temperature stress affects protein synthesis and conformation, causing protein damage (Hofmann and Somero 1995, 1996; Hochachka and Somero 2002; Rinehart et al. 2006; Todgham et al. 2007). Once the damaged protein exists, the regulation associated with the repair and degradation of damaged proteins is subsequently triggered to prevent increase in protein aggregation that is harmful to cell viability (Kabakov and Gabai 1993; Wickner et al. 1999; Goldberg 2003; Bukau et al. 2006). Activation of HSPs and protein ubiquitination which are involved in protein refolding and degradation in response to change in ambient temperature in aquatic animals have been reported in several previous studies (Hofmann and Somero 1995, 1996; Hochachka and Somero 2002; Hofmann et al. 2002; Place et al. 2004; Iwama et al. 2006; Todgham et al. 2007; Cui et al. 2011, 2013). However, the evidence of protein aggregation level was lack to address the PQC mechanism adequately. In the present study, HSPs and ubiquitin-conjugated proteins evidently elevated in *C. viridis* exposed to 32°C (Figures 1 and 2), whereas protein aggregation was similar to the normal temperature group at low level (Figure 3). However, elevated protein aggregation levels were found when organisms were cultured in high mortality conditions (Rinehart et al. 2006; Choe and Strange 2008). Thus, our findings assumed that the upregulation of HSPs and ubiquitin-conjugated proteins was sufficient to prevent the accumulation of aggregated proteins in *C. viridis* to adapt to transient

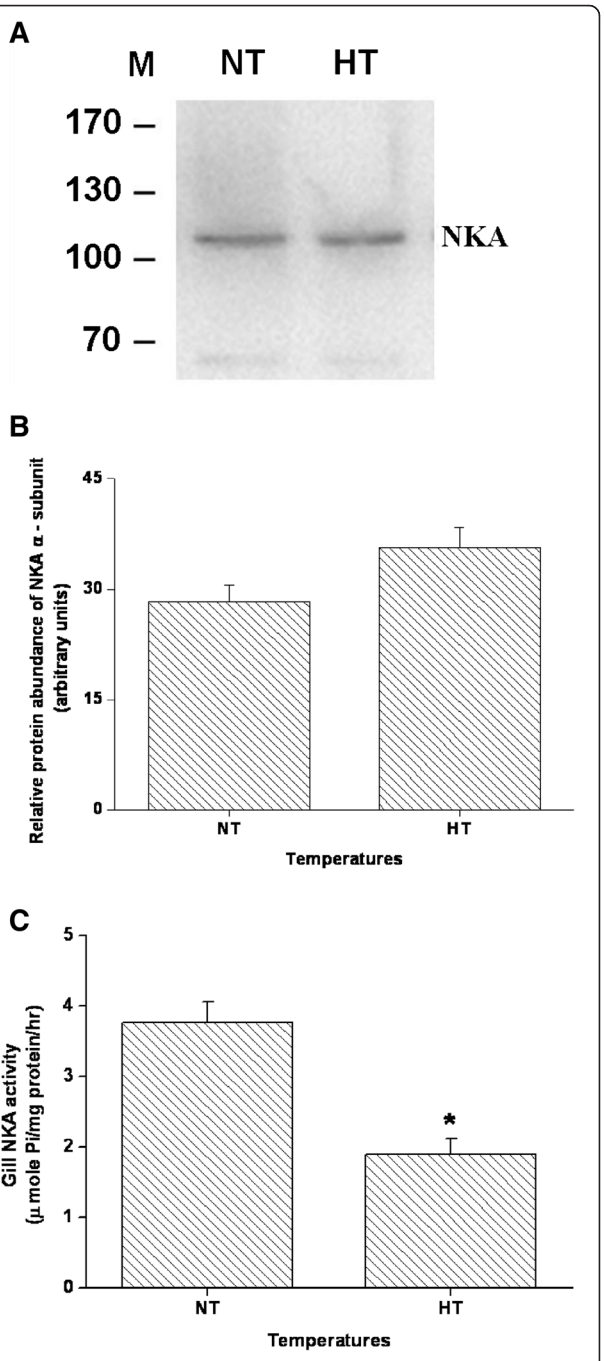

Figure 4 Impact of changes in temperature on the responses of gill Na^+/K^+-ATPase (NKA) in blue-green damselfish (*Chromis viridis*). (A) Immunoblots of *C. viridis* gills probed with a monoclonal antibody (α5; DSHB) to NKA α-subunit. The immunoreactive bands of HT group were more intensive than NT group. **(B)** Relative abundance of immunoreactive bands of NKA α-subunit in the gills of different temperature groups ($n = 5$). Expression of NKA α-subunit was similar between NT and HT groups. **(C)** Specific activity of gill NKA in *C. viridis* in response to elevation of ambient temperature ($n = 5$). Downregulation of branchial NKA activity was found after hyperthermic exposure. The asterisk indicated a significant difference ($P < 0.05$) by unpaired *t* test. Values were mean ± SEM. M, marker; NT, normal temperature (26°C); HT, high temperature (32°C).

elevation of ambient temperature. To our knowledge, this is the first study to examine the expression of HSPs, ubiquitinated proteins, and protein aggregation levels simultaneously in fish, in response to temperature challenge.

The internal ionic and osmotic balance of fish is affected by ambient temperature (Gonzalez and McDonald 2000; Metz et al. 2003; Sardella et al. 2004; Fiess et al. 2007; Sardella et al. 2008a, b). After exposure of *C. viridis* to hyperthermic condition, significant increase of plasma osmolality as well as decrease of muscle water content were found (Table 1). This might be explained by the marked depression of branchial NKA activity at 32°C, even though the protein expression of gill NKA was not affected (Figure 4). Moreover, in this study, NKA activity was assayed at the exposure temperature of the fish to show the apparent NKA activity to provide a physiological interpretation of our results. This is because temperature affects the reactivity of molecules by affecting protein conformation, kinetic properties, and assembly. On the other hand, activation of ion transporter system is energy-required while the rate of cellular respiration the main process for energy providing is temperature-dependent (Hochachka and Somero 2002). Therefore, the decrease in gill NKA activity reflected that metabolically-dependent ion transporter proteins are more susceptible to temperature change than is passive ion diffusion (Christensen 1975; Hochachka and Somero 2002). Furthermore, temperature inhibited the specific activity of NKA was found in the common carp (*Cyprinus carpio*) and the Mozambique tilapia (*Oreochromis mossambicus*). By using biochemical and immunohistochemical approaches, it was found that a lower apparent NKA activity was compensated for by strongly enhanced NKA expression (Metz et al. 2003; Sardella et al. 2008a). The present study was difficult to rule out the possibility that the other compensatory responses were enhanced in *C. viridis* only based on protein expression of gill NKA.

Conclusions

A local species and recorded in situ water temperature were used in this study to understand the impacts of short-term increases in temperature on stress responses, cellular protein stability, and osmoregulatory status in a reef-associated fish by using physiological and molecular approaches. The results provided the implication for elucidation that *C. viridis* possesses the molecular mechanisms for coping with thermal stress to maintain protein stability, but inhibitory effects on osmoregulatory ability resulted in slight changes of plasma osmolality and muscle water content.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

The work presented here was carried out in collaboration among all authors. CHT, MYL, and KS designed and carried out the experiments. CHT drafted the manuscript. LYH and WBC helped for rearing the experimental animals. All authors made comments on the manuscript. All authors read and approved the final manuscript.

Acknowledgements

The α5 monoclonal antibody were purchased from the Developmental Studies Hybridoma Bank (DSHB) maintained by the Department of Pharmacology and Molecular Sciences, John Hopkins University School of Medicine, Baltimore, MD 2120521205, and the Department of Biological Sciences, University of Iowa, Iowa City, IA 52242, under Contract N01-HD-6-2915, NICHD, USA. This study was supported by the grants from the National Science Council of Taiwan (NSC 102-2313-B-259-001 to C.H.T.) and the National Museum of Marine Biology and Aquarium (to C.H.T.).

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Received: 24 February 2014 Accepted: 3 April 2014
Published online: 29 April 2014

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doi:10.1186/s40555-014-0021-7
Cite this article as: Tang *et al.*: **Short-term effects of thermal stress on the responses of branchial protein quality control and osmoregulation in a reef-associated fish, *Chromis viridis*.** *Zoological Studies* 2014 **53**:21.