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Effect of water temperature on growth, survival and health status of East Asian Bullfrog (*Hoplobatrachus rugulosus*) Larvae

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Abstract: East Asian Bullfrog (*Hoplobatrachus rugulosus)* is an economically and nutritionally important animal in Thailand. However, many factors including UV radiation, infection and especially the fluctuation of temperature adversely affect the East Asian Bullfrog culture. Thus, the aims of this study was to investigate the effects of water temperature changes on the survival and growth as well as oxidative stress of the *H. rugulosus* tadpoles. The result showed that cold shock and heat shock significantly decreased the survival rate of the tadpoles when compared to those of control groups. Cold shock at 23°C showed the highest survival rates among the cold shocked groups and the heat shock at 27°C showed the highest survival rates among the heat shocked groups. However, there was no significant difference ($p>0.05$) of their body length at day 0 and day 7 among the experimental groups. Histological examination showed blood congestion and a large number of melanomacrophage and Kupffer cell in the liver tissue of tadpoles exposed to cold and heat shock when compared to those of the controls. Moreover, blood congestion was found in tadpoles exposed to the cold shock (17°C). In addition, cold and heat shock groups revealed significantly increased malondialdehyde (MDA) level in whole body of the tadpoles compared with the control group. Moreover, the level of MDA in the cold shock group was significantly greater than that in the heat shock group. The data obtained from this study will help to raise awareness of temperature shock in farm-raised frogs and others wild amphibian species.

Keywords: Growth, *Hoplobatrachus rugulosus*, Survival, Tadpole, Temperature.

Introduction

East Asian Bullfrog (*Hoplobatrachus rugulosus*, Wiegmann, 1835) is an anuran amphibian with a widespread distribution from central China, Myanmar, Peninsular, Malaysia including Thailand. They are commonly found in wetlands and paddy fields (Diesmos et al., 2004; Gratwicke et al., 2009). This species is economically important in Thailand because it is used as food by the local people and they are a very good source of protein. Moreover, frozen frog legs of farm-raised frogs are a part of the exports to many countries (Oza, 1990). However, they are susceptible to changes in their natural environment brought on, for example, by pollution, pesticides, diseases, especially the climate change.

Frogs are ectothermic animals, water temperature is one of the most important environmental factors affecting the physiology of organisms because they do not have a thermoregulatory mechanism (Goldstein et al., 2017). Earlier studies indicated that temperature fluctuation could influence their survival, growth and physiological functions in the wild and farmed frogs (Wang et al., 2012; Bellakhal et al., 2014). Moreover, Sahoo and Kara (2014) demonstrated that cold shock on Tiger frogs (*H. rugulosus*) caused acute mortality in the larvae. Thus, these phenomena were the main causes of the mortality and the decline in frog populations. In addition, Kern et al. (2015) found that water temperature fluctuation on Australian frogs (*Limnodynastes peronii*) inhibited growths and developments by significant decrease in total length (TL), body mass (Mb) and body length (BL) when compared with those reared in constant water temperature. Furthermore, temperature fluctuation can lead to oxidative stress in organisms by increased production of reactive oxygen species (ROS), which cause lipid peroxidation (Sahin, 2004). Sahoo and Kara (2014) reported that the exposure of short-term cold stress to common Indian toads (*Bufo melanostictus*) significantly increased ROS that was indicated by the rising of lipid peroxidation in the liver, kidney and brain tissues. Additionally, thermal stresses can also effectively induce histological alterations in the liver of other ectotherms, such as fat droplets accumulation (Liu et al., 2018), mitochondrial swelling (Xu et al., 2018), hepatocyte degeneration and necrosis (Ates et al., 2006). It has been reported that histological examination in the liver tissue of giant spiny frog *(Quasipaa spinosa)* exposed to cold and heat shock, erythrocyte extravasation as hemorrhage and congestion was observed. Inflammatory cell infiltration was conspicuous and enlargement of sinusoids was also presented (Liu et al, 2018). Therefore, the present study aimed to investigate the effects of water temperature changes on the survival and growth as well as oxidative stress of the *H. rugulosus* tadpoles.

Materials and Methods

Animals and experimental design

Experiments were performed with *H. rugulosus* larvae. Tadpoles, Gosner stage 26, were obtained from Thunpisit frog farm, Chiang Mai province. They were kept in dechlorinated water with laboratory conditions at least 24 hours for acclimation. The tadpoles were fed twice daily with commercial tadpole diet. Experimental designs were as followed:

Experiment 1 – The frog tadpoles were divided into 7 groups comprising of 30 tadpoles each. Group 1-3 were served as cold shock groups and groups 4-6 were served as heat shock groups. For cold shock groups, the initial temperature was set at 25°C and then decreased to 23°C, 20°C and 17°C within 4 hours. For heat shock groups, the initial temperature was set at 25°C and then increased to 27°C, 30°C and 33°C within 4 hours. The control group was maintained at 25°C throughout the experimental period. Each treatment was conducted in triplicate. Dead larvae, if present, were counted and removed daily. After 7 days of exposure, the survived tadpoles were measured for their body length to determine the growth rate. The liver tissues of frog tadpoles were collected and processed for histological investigation.

Experiment 2 – The frog tadpoles were divided into 3 groups comprising of 30 tadpoles each. Group 1 was served as control group and the temperature was set at 25°C. Group 2 was set as cold shock group and the temperature was started at 25°C and then decreased to 23°C (the temperature which was produced the highest viability of cold shock-exposed larvae from the experiment I). Group 3 was heat shock group and the temperature was started at 25°C and then increased to 27°C (the temperature which was produced the highest viability of heat shock-exposed larvae from the experiment I). Each treatment was conducted in triplicate. The tadpoles were exposed to heat and cold temperatures until they reached the Gosner stage 42. They were then collected for determining oxidative stress by evaluating the level of malondialdehyde (MDA).

Histopathological examinations

The tadpoles at day 7 were collected and whole body were fixed in Bouin's solution. After fixation for 24-30 h, tissues were dehydrated through a graded series of ethanol, cleared in xylene, and then embedded in paraffin. Six µm sections were made and stained with hematoxylin and eosin (H&E). The sample was analyzed by assessing the histopathological alterations under a light microscope.

Estimation of lipid peroxidation in tadpoles tissue.

Twenty milligrams of homogenated tadpoles tissue were mixed in 1 ml of 0.1 M Phosphate Buffer Solution (PBS), pH 7.4 at -4 °C. The mixed sample was centrifuged at 1,155 x g for 10 minutes and the supernatant was stored at -40°C for examination of MDA within 24 hours. The degree of lipid peroxidation was determined colorimetrically using the method of Buege and Aust (1976). Briefly, 100 µl of sample was mixed with 450 µl of normal saline, 200 µl of thiobarbituric acid (TBA) and 1,000 µl of trichloroacetic Acid (TCA). The mixture was heated at 100°C for 30 minutes, cooled with running tap water and then added with 2,000 µl of distilled water, mixed with vortex mixer and centrifuged at 1,155 x g for 10 minutes. The absorbance at 532 nm was then measured and calculated for MDA concentration against the MDA standard curve.

Estimation of protein in tadpoles tissue.

The level of protein content from each tissue was estimated following the method described by Bradford (1976) and the bovine serum albumin (BSA) was used as a standard protein. Five milligrams of Coomassie Blue G were dissolved in 1 ml of 25% (v/v) methanol containing 42.5% (v/v) H_3PO_4. One volume of the prepared dye was diluted with four volumes of distilled water, and 0.04 mL of each sample was mixed with 2 mL of diluted dye. The absorbance was read at 590 nm and calculated for protein concentration against the protein standard curve.

Statistical Analyses

All statistical analyses were managed with Statistical Package for Social Science (SPSS) software version 20.0 for Mac OSX (SPSS Inc., IBM). Values were verified for normality and homogeneity of variance by Shapiro-Wilk test and Levene's tests, respectively. Mean comparisons were carried out by one way analysis of variance (ANOVA) followed by Tukey's HSD test.

Result & Discussion

Survival and growth of tadpoles

As shown in Figure 1, cold shock and heat shock groups revealed significantly decreased survival rate of the tadpoles when compared to those of the control groups ($p<0.05$). Moreover, the survival rate of tadpoles in cold shock groups was not significantly different from that of heat shock groups. The highest survival rates in cold and heat shock were found at 23°C and 27°C, respectively. However, there was no significant difference ($p>0.05$) of their body length at day 7 among the experimental groups. (Figure 2) The highest survival rates obtained from our study were slightly associated with the studies of Wang et al. (2012) which reported that the mortality rate of

Tiger frog (*H. rugulosus*) exposed to cold shock were 28.1% at 12°C, 87. 5% at 10°C and 100% at 8°C. Additionally, Bellakhal et al. (2014) reported that African green frog (*Pelophylax saharicus*) survival was reduced at temperatures above 28 °C. These inferred that the temperature fluctuation on the survival rate among species may be determined by differences in their thermal optimum for survival. The larval tadpole *H. rugulosus* used by Wang et al. and *P. saharicus* used by Bellakhal might have greater thermal optimum range than the older larvae used in our study. In addition, the body length of tadpoles obtained from our study are contradiction with the studies of Kern et al. (2015) which, reported that body length of *L. tasmaniensis* tadpoles raised in temperature shock was significantly lower than those of tadpoles in the constant temperature. As a result, tadpoles obtained from cold and heat shock had reduced survival when compared with tadpoles reared in the constant temperature since the temperature has been well known as the most pervasive abiotic factor to influence physiological function because of thermodynamic effects on biochemical reactions which underline survival and growth (Somero, 2002). Environmental temperature determines body temperature in most ectotherms (Guderley, 2004; Seebacher and Murray, 2007). Consequently, the temperature fluctuation could affect the physiology, survival and growth of tadpole as seen in our study.

Histopathological changes in tadpole liver

Histopathological changes of liver were observed in all treated group (Figure 3). A larger number of melanomacrophage and Kupffer cells were observed in the liver tissue of tadpoles exposed to cold and heat shock Moreover, blood congestion was also found in the liver of the tadpoles exposed to the cold shock (17°C) (Figure 3D).

Our results were consistent with the studies of Corsaro et al. (1990) which reported that melanomacrophages in *R. esculenta* increased during the winter (about 5–10°C) and decreased during the summer (about 20°C –25°C). The liver has long been well known to be the main organ for detoxification that could suffer serious morphological alterations in tadpoles exposed to temperature shock. Therefore, its histological changes could be used as biomarkers for environmental stress (Sichel et al., 1987). The hematopoietic organs of ectothermic animals commonly display pigmented cells with phagocytic activity called melanomacrophage (Barni et al., 1999). They are focal accumulations of pigmented macrophages in the liver. These may contain four types of brown to black pigments, namely melanin, lipofuscin, ceroid, and hemosiderin/ferritin, which absorbs and neutralizes free radicals, cations, and other potentially toxic agents derived from the degradation of phagocytized cellular material. Thus, it appears that these cells are sensitive to stimuli as a way to adapt to stress conditions (Sayed, et al., 2015). In addition, Kupffer cells are specialized resident macrophages in the liver and the activated kupffer cells appears to modulate acute hepatocyte injury and chronic liver responses. They constitute the macrophage population of the body to encounter the bacteria which derived from the gastrointestinal tract and transported to the liver via the portal vein. Thus, the increase in the number of Kupffer cells in the liver tissue might be the response of the defense system when animals are in contact with environmental stress. Moreover, it has been reported that blood congestion was present in the liver tissues of giant spiny frog (*Q. spinosa*) under cold and heat stresses (Liu et al., 2018) which was consistent with our results. It could be attributed to the destruction of hepatic erythrocytes and newly produced erythrocytes accumulated in the liver (Maekawa et al., 2012).

Lipid peroxidation in tadpole tissue

Biochemical analysis of tadpole tissue in the present experiments indicated that cold shock (23°C) (0.079±0.009 mM/mg protein) and heat shock groups (27°C) (0.050±0.002 mM/mg protein) induced a significant increase in MDA level compared with the control group. (0.021±0.006 mM/mg protein). Moreover, the level of MDA in the cold shock group (0.079±0.009 mM/mg protein) was significantly greater than those in the heat shock group (0.050±0.002 mM/mg protein) (Figure 4).

This is in agreement with the findings of previous studies of Sahoo and Kara (2014) which report that MDA level in the liver, kidney and brain tissues of the common Indian toad (*B. melanostictus*) exposed in cold and heat shock were higher than those of the tadpoles exposed in constant temperature. In addition, the level of MDA of the liver of goldfish (*Carassius auratus*) obtained in heat shock was higher than that was obtained in the constant temperature. As a result, temperature change induces a significant increase in the MDA level in tissue of ectotherm animals. (Abele et al., 2002). It is commonly known that the MDA is one of the important biochemical compounds used as an indicative of reactive oxygen species (ROS) being generated from lipid peroxidation. In general, ROS, which can be neutralized by a variety of antioxidants, are generated by cellular metabolism (Lushchak and Bagnyukova, 2006). However, excessive ROS would damage various chemical and biological membranes and it has been suggested as a cause of toxicity in several organs. Furthermore, oxidative stress has also been shown to affect organ dysfunction and tissue damage. The cold and heat stress, which induces elevated thermogenesis by enhanced substrate combustion along with increased oxygen consumption, has been reported to produce ROS, which cause lipid peroxidation and play an important role in tissue injury (De Quiroga et al., 1991).

Conclusion

In summary, we concluded that the temperature change caused decrease survival and growth of East Asian Bullfrog (*H. rugulosus).* In addition, temperature shock caused oxidative stress that was demonstrated by the increased MDA level and triggered the histopathological alterations in the livers of *H. rugulosus* tadpoles. It could be suggested that the data obtained from this study would help to raise awareness of temperature shock in farm-raised frogs and others wild amphibian species.

Acknowledgement

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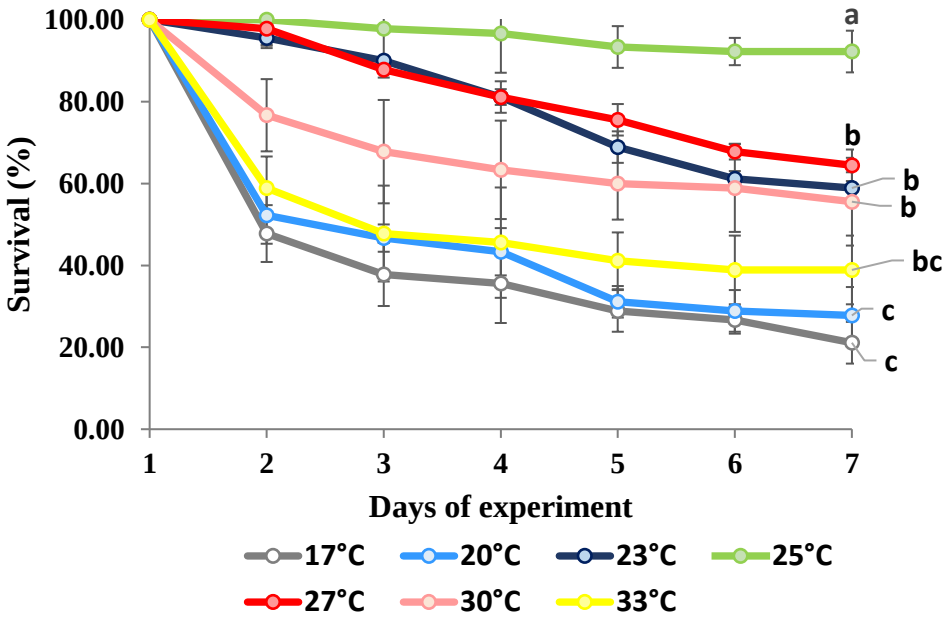

Figure 1. Survival of *H. rugulosus* tadpoles exposed to cold shock (23°C, 20°C, 17°C) and heat shock (27°C, 30°C, 33°C) for 7 days as compared to control group (25°C) (Difference in labelling letter (a, b, c) represents significant difference at $P < 0.05$).

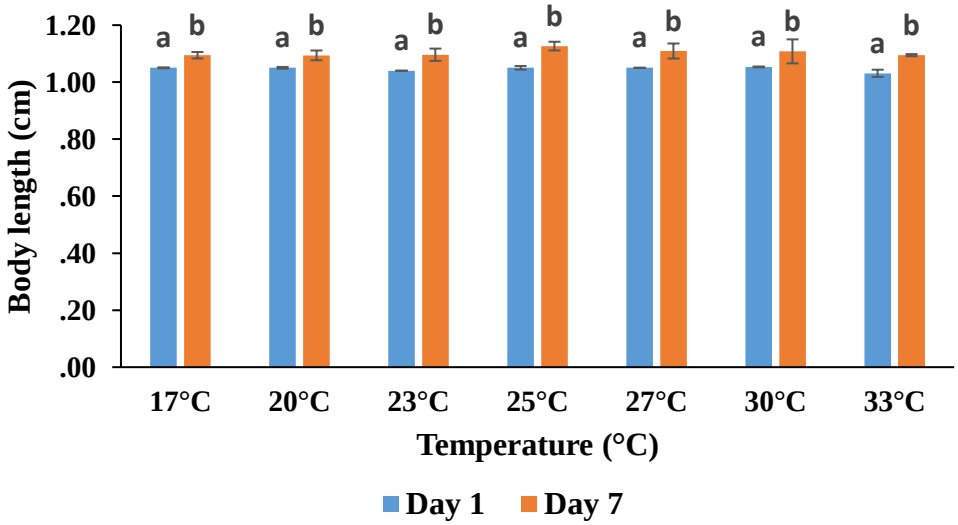

Figure 2. Body length of *H. rugulosus* tadpoles exposed to cold shock (23°C, 20°C, 17°C) and heat shock (27°C, 30°C, 33°C) for 7 days as compared to control group (25°C) (Difference in labelling letter (a, b) represents significant difference at $P < 0.05$)

Control group

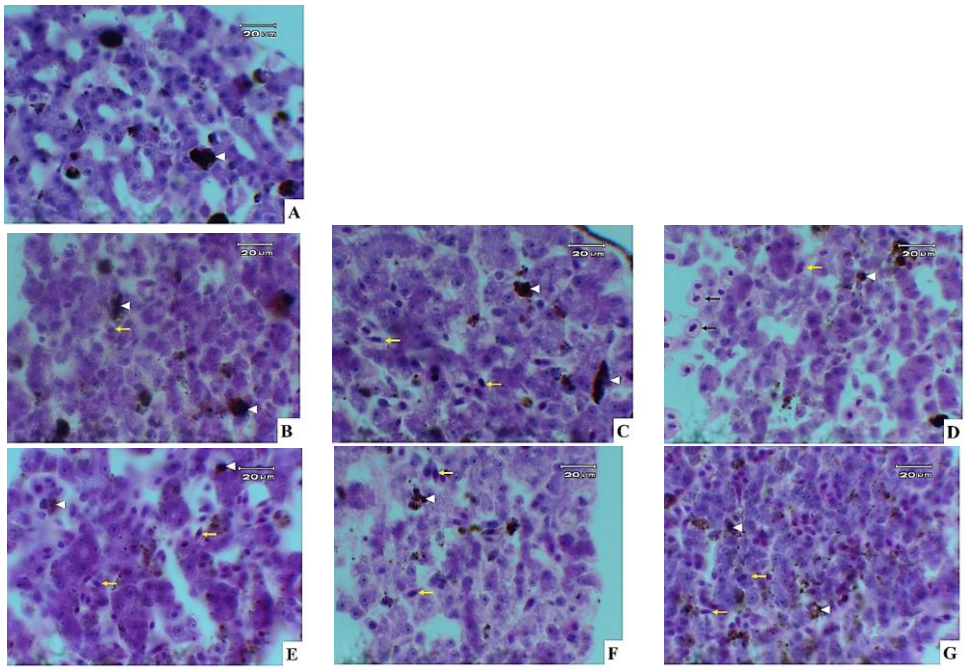

Figure 3. Liver tissue of *H. rugulosus* tadpoles (A) control; (B) exposed to cold shock (23°C); (C) exposed to cold shock (20°C); (D) exposed to cold shock (17°C); (E) exposed to heat shock (27°C); (F) exposed to heat shock (30°C); (G) exposed to heat shock (33°C); white arrowhead : melanomacrophage; yellow arrow : Kupffer cell; black arrow : blood congestion; H&E. x200.

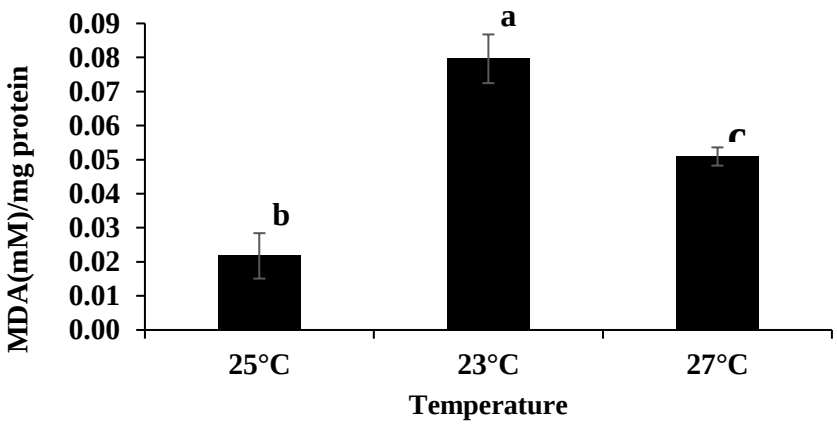

Figure 4. The level of malondialdehyde (MDA) of *H. rugulosus* tadpoles exposed to cold shock (23°C) and heat shock (27°C) for 7 days as compared to control group (25°C) (Difference in labelling letter (a, b, c) represents significant difference at $P<0.05$.)