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碩士論文

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鹽度對生活在半淡鹹水蝌蚪高溫耐受度的影響
Effect of salinity on the critical thermal maximum
of tadpoles living in brackish water

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致謝

真不敢相信我能完成研究跟論文撰寫!這一趟旅程幾經挫折，數次試圖放棄，但最終還是撐了過來，多虧身邊的老師同學以及家人的鼓勵。首先必須感謝關永才老師，我在研究所的這一兩年，慢慢感受到關老師對我的影響跟自己的成長。我總是狀況外，要不是有關老師的鞭策，我絕對無法獨立並準時完成研究，而且我初次嘗試英文寫作，也讓老師吃了不少苦頭，心裡真得很抱歉但也很高興能有這個機會嘗試更大的一步並得到更多的收穫。我同時很感謝老師，如此關心我的生活狀況，給予我很大的幫助，給了我很多的機會跟資源，讓我能專心的完成學業，之後也能順利就業。我也很感謝我的口試委員周文豪老師、巫奇勳老師，和張原謀老師。看了周老師在我的初稿中認真的註記真的很感動，感謝老師百忙之中研讀了我的論文，仔細的檢察我的格式及文獻，對於粗心的我來說真的幫忙很大，而且給了我很多建議是必須要好好思考的，也提醒我自己不要理所當然了自己熟知的事，還是要以更客觀的角度來檢視每一個理論。我也謝謝巫老師在我大四以來對我的照顧，因為老師跟我的實驗室親緣關係較近，所以很多問題我都首先去煩老師，老師也帶我去出野外好幾次，並介紹吳順源老師帶我在雲林出野外好幾次，真的很感謝吳老師。巫老師也拿了很多文獻給我讀給我很多方向，對於剛接觸這個研究的我來說幫助真的很

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中文摘要

全球暖化造成全球棲地溫度升高，以及海平面上升導致棲地鹽化等環境問題對沿海兩棲類族群造成巨大的衝擊。許多研究探討兩棲類蝌蚪在逐漸升溫下的溫度生理，以及在鹽度壓力下的生理反應。然而，鮮少研究結合溫度及鹽度兩個因子並檢驗蝌蚪在不同鹽度條件下的溫度生理。在此研究，我探討蝌蚪的高溫耐受度是否會受到環境鹽度的影響而變動。我於台南城西里防風林採集澤蛙及小雨蛙的蝌蚪，並於雲林的半淡鹹水棲地採集黑眶蟾蜍的蝌蚪。將蝌蚪馴養在三種處理組，分別為淡水、低鹽度及高鹽度處理組中七天，並於第八天進行高溫耐受度測量。另外，我在實驗期間內也觀察了存活與發育。結果發現，澤蛙及黑眶蟾蜍的蝌蚪，在高鹽度處理組中高溫耐受度明顯低於低鹽度及淡水處理組，而三種受試蝌蚪在測量結果中都展現高溫耐受度隨著鹽度上升而降低的一致趨勢。此外，蝌蚪生存率與發育在高鹽度處理下也有與高溫耐受度相似的趨勢。我推測蝌蚪高溫耐受的下降和蝌蚪減少攝食、體內能量分配及/或脫水有關。本研究結果對全球暖化如何影響沿海蛙類有重要意義。暖化現象可能對沿岸蛙類有雙重的影響。全球暖化造成溫度上升及沿岸棲地鹽化，環境溫度上升逐漸接近動物的高溫耐受，威脅動物存活;在另一方面，海平面上升造成沿岸棲地鹽化，鹽度使蝌蚪高溫耐

受降低，使之在高溫度下更加脆弱。因此，全球暖化對蛙類存活及數量的負面影響可能比以前想像的還嚴重。

關鍵字:全球暖化, 棲地鹽化, 兩棲類, 溫度生理, 壓力



Abstract

Global warming is considered to be a main cause of rising ambient temperature as well as sea-level which results in habitat salinization, and both of these issues could impact on coastal amphibian populations. Earlier studies have investigated the thermal physiology of amphibian tadpoles in elevated temperatures and physiological responses to salinity stress. However, few study has assessed the combined effects of these factors and examined the thermal physiology of tadpoles under different salinities. The aim of the study was to assess the critical thermal maximum (CT_{max}) of tadpoles in varying salinity conditions. I collected tadpoles of *Fejervarya limnocharis* and *Microhyla fissipes* in windbreaks in Chung-Xi village in Tainan and *Duttaphrynus melanostictus* in the brackish habitats in Yunlin. Tadpoles were reared in freshwater and low and high salinity treatments for 7 days and CT_{max} was measured. Besides, the survival and development of tadpoles were also recorded throughout the study period. Results showed that the CT_{max} of *F. limnocharis* and *D. melanostictus* tadpoles in high salinity treatments were significantly lower than other treatments, and all test species showed a consistent pattern that the CT_{max} declined with the

elevated salinity. Survival and development of tadpoles both showed similar patterns among treatments. My finding of a decline of CT_{max} in high salinity agrees with results of earlier studies on marine animals. The possible mechanism for the decline of CT_{max} in high salinity is due to energy acquisition and allocation and/or dehydration response. My finding of CT_{max} of tadpoles declined in high salinity treatments has important implication on the effects of global warming on coastal frog populations. My results imply that global warming has double impacts on the biology of frogs. During the larval period, rising temperature due to global warming on one hand gradually approaches the upper thermal limits of tadpoles which threatens their survivorship, on the other hand causes sea level to rise which result in habitat salinization of aquatic habitats in coastal areas. The salinity stress retards the growth and development and reduces the ability of tadpoles to tolerate high temperature. Together, the negative effect of global warming on the survival and abundance of frogs might be more serious than we originally thought.

Keywords: Global warming, habitat salinization, amphibians, thermal physiology, stress

Introduction

Global warming is a major artificial consequence associated with the increasing temperature which leads to the thermal expansion of seawater as well as melting of polar ice cap and dominating the rising of global mean sea level (Root et al. 2003; Meehl et al. 2005; IPCC 2013; Kopp et al. 2014). Measured global mean sea level rise from late 19th century to early 21st century is 195 mm on an average velocity of 1.44 mm yr⁻¹ (Church and White 2006; 2011). In addition, the global mean sea level which is corresponding to rising global temperature was projected to uplift for 1m or more by the end of 21st century corresponding to the rapidly rising temperature in a climate model (Vermeer and Rahmstorf 2009; Horton et al. 2013). Changes in sea level scenario will lead to ecological impacts such as reduction in suspended sediment in estuaries, habitat loss, and salinization in coastal areas and affect coastal ecosystem (Day et al. 1995; Feagin and Sherman 2005; Nicholls 2011).

Changes in environmental factors such as temperature and salinity can have great impacts on animals, especially amphibians. Amphibians occupy a great variety of habitats and depend on both aquatic and terrestrial habitats (Boutilier et al. 1997). Frogs are sensitive to

environmental changes for their permeable skin and poor ability in osmoregulation (Boutilier et al. 1997). Moreover, frogs have a complex life cycle including aquatic larvae and terrestrial adults, and all life history stages highly depend on the availability of freshwater and humid environments (Shoemaker and Nagy 1977). Besides, tadpoles are more vulnerable to changes in environmental factors than adults because they are restricted in aquatic habitats in which they develop, making them more likely to perish in environments with a bad water quality (Viertel 1999).

Rising water temperature is challenging tadpoles with their thermal tolerance and affecting their survival and development. Thermal tolerance varies greatly among species (Deutsch et al. 2008; Duarte et al. 2012). The critical thermal maximum (CT_{max}) is commonly applied as an index to assess the sensitivity of organisms to high temperature (Licht and Brown 1967; Feder and Pough 1975; Keen and Schroeder 1975; Hoppe 1978; Cupp 1980; Howard and Wallace 1983; Floyd 1983, 1985; Manis and Claussen 1986; Layne et al. 1987; Lutterschmidt and Hutchison 1997). Earlier studies have evaluated the impact of warming temperature on species across latitudes using the warming tolerance (WT)

as an index, which is defined as the differences between CT_{max} and the highest habitat temperature (T_{max}) (Deutsch et al. 2008; Duarte et al. 2012). Results of earlier studies suggest that impacts of rising temperature are more severe on species live in tropical areas due to the narrower WT when compared to temperate areas (Deutsch et al. 2008; Duarte et al. 2012; Simon et al. 2015). In addition, species with lower CT_{max} exposed to a greater risk in warming ambient temperature because of the smaller WT (Deutsch et al. 2008; Simon et al. 2015).

Apart from rising temperature, rising sea level results in habitat salinization which negatively affects coastal amphibians physiologically. Earlier studies have reported that salinity exposure may decrease the survival and development of tadpoles (e.g. *Bufo calamita*, *Litoria aurea*, *Fejervarya limnocharis*, *Incilius nebulifer*, *Bufo balearicus*, *Bufo bufo*, *Litoria ewingii*; Gomez-Mestre and Tejedo 2003; Christy and Dickman 2002; Wu and Kam 2009; Alexander et al. 2012; Wu et al. 2012; Bernabò et al. 2013; Kearney et al. 2014). Exposing to salinity could induced the branchial Na⁺/K⁺-ATPase (NKA) expression for osmoregulation in tadpoles (Bernabò et al. 2013; Wu et al. 2014) as well as dehydration response in a cellular level (Wu et al. 2014). Tadpoles of some frog

species are more sensitive to salinity (Christy and Dickman 2002), and variations of salinity tolerance among different amphibian species directly influence the community composition, species abundance, and distribution (Brown and Walls 2013).

Frog species or populations that live and breed in coastal zones may be more severely affected by a global warming scenario. Frogs generally avoid inhabiting and breeding in brackish habitats (Balinsky 1981; Duellman and Trueb 1994) since water salinity is an environmental stressor to them due to their permeable skin and poor ability in osmoregulation (Boutilier et al. 1997). Nevertheless, field surveys have revealed that some frog populations still breed in brackish water (Alcala 1962; Gordon and Tucker 1965; Dunson 1977; Uchiyama et al. 1990; Wu and Kam 2009). In face of global warming, frogs are subjected to heat threat due to increasing ambient temperature. Moreover, global warming leads to habitat salinization caused by rising sea level may also affect their thermal tolerance. Earlier studies have demonstrated that CTmax decreased in high salinity in marine animals such as yellow-fin sea breams (*Acanthopagrus latus*), green sturgeon (*Acipenser medirostris*), and Antarctic collembolan (*Cryptopygus antarcticus*) (Jian

et al. 2003; Sardella et al. 2008; Everatt et al 2013). However, most studies mainly focused on marine ectotherms, it is relatively unknown whether salinity affects thermal tolerance of ectotherms in freshwater habitats. In view of amphibian is an indicator to habitat conditions and tadpoles are specifically sensitive to water quality changes, they can be a good model to study on thermal physiology in a salinity stress.

In this study, I used three frog species (*Fejervarya limnocharis* (Dicroglossidae), *Duttaphrynus melanostictus* (Bufonidae) and *Microhyla fissipes* (Microhylidae)) that breeding in coastal brackish habitats as the study animals to assess the effect of salinity on the thermal tolerance of tadpoles. Specifically, I studied the CTmax of tadpoles which acclimated to different salinity conditions. Based on the findings that salinity has detrimental effects on thermal tolerance of vertebrates and invertebrates (Jian et al. 2003; Sardella et al. 2008; Everatt et al 2013), I predicted that the CTmax of tadpoles would decrease as salinity increased.

In addition, earlier findings indicated that salinity may decline the survival and development rate of tadpoles (Christy and Dickman 2002; Wu and Kam 2009; Alexander et al. 2012; Wu et al. 2012; Bernabò et al. 2013; Kearney et al. 2014), I also predicted that the survival and

development of tadpoles would be negatively affected by salinity.



Materials and methods

Study animals and study sites

Tadpoles of *F. limnocharis*, *D. melanostictus* and *M. fissipes* were used in this study. They are common species in lowland and breed from spring to summer. Typical breeding habitats include streams, pools, swamps, and rice paddies. However, some populations breed in brackish habitats in coastal areas. Animal collection was conducted in the summer of 2016, and tadpoles of *F. limnocharis* and *M. fissipes* were collected from the windbreak woodlands in Cheng-Xi village of Tainan County (Chang et al. 2016). The brackish tidal gullies where tadpoles were found were well covered with leaves and branches of *Casuarina equisetifolia*. The salinity fluctuation in tidal gullies depends on the rainfall and evaporation. Occasionally, when typhoon comes, high tides bring seawater into those tidal gullies where salinity dramatically increases from about 1 up to 8 ppt. Tadpoles of *D. melanostictus* were collected from Lim-Chu in the Yun-Lin County. Brackish water in this area is caused by the seawater intrusion into groundwater caused by land subsidence (Peng et al. 2005).

Experimental design

1. Salinity acclimation

I collected 30-50 tadpoles at Gosner stage 26-30 for each species (Gosner 1960) and brought back to the laboratory. Three different treatments (freshwater, low salinity, and high salinity) were used, and the salinity setting was depended on their maximum salinity tolerance. For *F. limnocharis* and *D. melanostictus*, the low and high salinity treatments were 4 and 8 ppt, respectively (Wu and Kam 2009; Karraker et al. 2010). For *M. fissipes*, the low and high salinity treatments were 3 and 5 ppt, respectively. The treatments set for *M. fissipes* were based on a preliminary experiment on salinity tolerance of tadpoles (Supplemental Information). Tadpoles were randomly assigned to three treatments (n=10-15), each tadpole was reared in a 10.5 × 7.5 × 4.5 cm of plastic container filled with 150 ml corresponding solution. The freshwater solution used in this study was tap water, and the salinity solution was the mixture of deionized water and Coralife sea salt (Energy Savers Unlimited, INC, Carson, CA, USA). Salinity was measured using an electronic salinity meter (Rixen brand, Model SM-10, Seoul, South Korea) under room temperature. Tadpoles were placed in an incubator which

was kept at 25°C and with a 12D:12L photoperiod.

2. Effect of salinity on the tadpole survival and development

The acclimation was lasted for a total of 7 days. Water was changed every day to prevent the deterioration of water quality. Tadpoles of *F. limnocharis* and *D.melanostictus* were fed with cooked vegetable, and tadpoles of *M. fissipes* were fed with fine fish chow daily. Survival of tadpoles in each treatment was recorded every day. Developments of tadpoles were measured before and after salinity acclimation (Wu et al. 2012).

3. The critical thermal maximum measurement

The critical thermal maximum (CT_{max}) of tadpoles was measured at the eighth day of acclimation, which is defined as “a certain thermal heat point that an animal losing its ability to escape or normally active” (Lutterschmidt and Hutchisons 1997). Each tadpole that survived during salinity acclimation was put into a beaker filled with 200ml treatment water. The beaker was then put into a plastic box filled with water and then into a temperature-control water bath. Water was heated

from ca. 25°C at the rate of 0.25°C/min (Wu and Kam 2005). I used a blunt probe to assess the activity of the tadpole. I determined the CTmax when a tadpole became disorganized in locomotion and lost its ability to escape from the stimuli. I tested 12 tadpoles at most for each treatment in each species. Right after testing, tadpoles were transferred into cool water immediately for recovery. Those tadpoles which didn't recover from the heat stress were excluded from further data analyses.

Statistical analyses

The survival of tadpoles during the acclimation was analyzed with survival analysis (Cox proportional hazard model and Kaplan–Meier survival curve). Kruskal-Wallis test with Dunn posterior test were used for comparison between the developments and CTmax of tadpoles acclimated in different treatments. Data analyses were conducted using the R i386 3.1.0 software. Significant level was set as $\alpha=0.05$.

Results

Effect of salinity on survival and development

The survival analysis showed that in *F. limnocharis*, *D. melanostictus*, and *M. fissipes*, survival of tadpoles was significantly different among treatments (Cox model, likelihood ratio = 13.6, $df = 2$, $p = 0.001$, likelihood ratio=20.4, $df = 2$, $p < 0.001$, and likelihood ratio = 9.12, $df = 2$, $p = 0.011$, respectively; Fig. 1). There was no mortality occurred in freshwater and low salinity treatments in three species. In the high salinity treatments, *F. limnocharis*, *D. melanostictus*, and *M. fissipes* survived 57, 60, and 64% of tadpoles at the end of salinity acclimation, respectively (Fig. 1). For all three species, development of each species before acclimation was not different among treatments (Kruskal-Wallis test, *F. limnocharis*: $\chi^2 = 0.69$, $df = 2$, $p = 0.71$; *D. melanostictus*: $\chi^2 = 1.38$, $df = 2$, $p = 0.502$; *M. fissipes*: $\chi^2 = 4.04$, $df = 2$, $p = 0.133$) (Fig. 2). For *F. limnocharis*, there were significant differences in development among treatments after acclimation (Kruskal-Wallis test, $\chi^2 = 19.31$, $df = 2$, $p < 0.001$) (Fig. 2A). Post hoc comparisons showed that development of tadpoles in freshwater was significantly higher than tadpoles in low ($p = 0.001$) and high salinity treatment ($p < 0.001$), and development of

tadpoles in low salinity treatment was also significantly higher than in high salinity treatment ($p = 0.008$) (Fig. 3A). Similarly, there were significant differences among treatments in development of *D.*

melanostictus tadpoles (Kruskal-Wallis test, $\chi^2 = 16.14$, $df = 2$, $p < 0.01$)

(Fig. 2B). Post hoc comparisons showed that there was no significant

difference between freshwater and low salinity treatments ($p = 0.285$),

and tadpoles in high salinity treatment were significantly lower than

tadpoles in freshwater ($p < 0.001$) and low salinity treatment ($p < 0.001$)

(Fig. 3B). In contrast, there was no significant difference among

treatments in development of *M. fissipes* tadpoles (Kruskal-Wallis test, $\chi^2 = 19.31$, $df = 2$, $p = 0.6$) (Fig. 3B and Fig. 3C).

Tadpoles acclimated in freshwater or low salinity generally consumed food and left little food debris, and tadpoles' excrement can be seen in the water. However, tadpoles that acclimated in high salinity were observed to fed less, and the majority food were uneaten.

Effect of salinity on CTmax

Results showed that there were significant differences of CTmax among treatments in both *F. limnocharis* and *D. melanostictus* tadpoles

(Kruskal-Wallis test, $\chi^2 = 26.6$, $df = 2$, $p < 0.001$; $\chi^2 = 20.58$, $df = 2$, $p < 0.001$, respectively). Post hoc comparison showed that CTmax of *F. limnocharis* tadpoles in high salinity treatment was significantly lower than in the low salinity treatment ($p = 0.008$) and freshwater treatment ($p < 0.001$). Similarly, CTmax of tadpoles in low salinity treatment was significantly lower than in freshwater treatment ($p = 0.001$) (Fig. 4). There were consistent results in tadpoles of *D. melanostictus* where CTmax of tadpoles in high salinity treatment was significantly lower than in low salinity treatment ($p = 0.004$) and freshwater treatment ($p < 0.001$), and CTmax of tadpoles in low salinity treatment was significantly lower than in freshwater treatment ($p = 0.015$) (Fig. 5). In contrast, I did not find the significant effect of salinity on CTmax of *M. fissipes* tadpoles (Kruskal-Wallis test, $\chi^2 = 2.86$, $df = 2$, $p = 0.24$) (Fig. 6), but the trend was similar to the results of former species. In summary, all tested species showed a consistent pattern that CTmax of tadpoles decreased as salinity elevated.

Discussion

Effect of salinity on survival and development

Survival and development of tadpoles were lower in high salinity treatments, suggesting salinity have negative effects on tadpole fitness. These results were similar to the findings from earlier studies. An earlier study in *F. limnocharis* showed that tadpoles exhibited a lower survival and delayed development when salinity reached 9 ppt (Wu and Kam 2009). Similar results were reported in *Litoria ewingii* in that tadpoles showed a significant increased mortality and retardation growth when salinity was up to 5 ppt seawater (Chinathamby et al. 2006). Christy and Dickman (2002) reported that in *Litoria aurea* tadpoles, mortality increased when exposing in the salinity threshold between 2 ppt and 3 ppt seawater, and salinity stress caused low growth rates in tadpoles. Delaying development in tadpole stage could result in a greater risk of predation and drying up of aquatic habitats (Metcalf and Monaghan 2001). In this study, the development of *F. limnocharis* and *D. melanostictus* tadpoles in high salinity was significantly lower than that in freshwater, whereas there was no significant difference in tadpoles of *M. fissipes* among treatments, nevertheless, I found the similar trends in all

test species. In my preliminary study, *M. fissipes* has a lower salinity tolerance, proposing a lower salinity tolerant threshold. Furthermore, *M. fissipes* may have smaller range of salinity tolerance, making them more vulnerable in high salinity.

Delayed development in high salinity may due to energy acquisition and allocation. Sanzo and Hecnar (2006) observed that tadpoles of *Rana sylvatica* became less active and fed less in saline water. Earlier studies have shown that development of amphibian larvae can be affected by low food availability (Berven and Chadra 1988; Kupferberg 1997; Wright et al. 1999; Beachy et al. 1999). In this study, I observed all three species of tadpoles in high salinity treatments fed less than those in low salinity and freshwater treatments, suggesting foraging activity is affected by salinity, and thus retard development of tadpoles. On the other hand, energy allocation in salinity conditions may also affect the development of tadpoles. Gomez-Mestre et al. (2004) reported that tadpoles of *Bufo calamita* consumed more glucose in high salinity, suggesting tadpoles spend more energy in osmoregulation in high salinity. Earlier studies reported that exposing to salinity stress in tadpoles induced the branchial Na^+/K^+ -ATPase (NKA) expression to maintain osmolarity

homeostasis (Bernabò et al. 2013; Wu et al. 2014) which was energy expensive (Boonkoom and Alvarado 1971). In amphibians, the branchial NKA, which highly expresses in both hypo or hyperosmolarity environments, is the major enzyme in active transportation of ions (Boonkoom and Alvarado 1971). In addition, an earlier study reported that salinity depresses the thyroid hormone in tadpoles (Gomez-Mestre et al. 2004) which is an essential hormone that affects tadpole development (Dent 1988; Gomez-Mestre et al. 2004; Sherwood et al. 2005), thus, depression of the thyroid hormone in tadpoles due to high salinity may delay development of tadpoles. In the present study, both energy acquisition and energy allocation could contribute the negative effects of salinity on the development and possibly growth of tadpoles.

Effects of salinity on CT_{max}

The critical thermal maximum of tadpoles was lower when exposing to high salinity, suggesting salinity reduces their ability to tolerate heat stress. The CT_{max} of *F. limnocharis* and *D. melanostictus* tadpoles that acclimated in high salinity was significantly lower than those in freshwater treatment, while there was no significant effect of salinity

treatments on *M. fissipes* tadpoles. Nevertheless, all species showed a consistent pattern that CTmax declined with the elevated salinity concentrations. My findings agree with results from earlier studies in marine animals. Everatt et al. (2013) reported that CTmax of Antarctic collembolan (*Cryptopygus antarcticus*) acclimated in freshwater was 22.2°C but dropped to 18.9 °C when acclimated in 200 ppt. In addition, Jian et al. (2003) reported that CTmax of yellow-fin sea bream (*Acanthopagrus latus*) maintained at 33 ppt were significantly lower than in 0.3 and 15 ppt. Sardella et al. (2008) reported that CTmax of green sturgeon (*Acipenser medirostris*) that acclimated in bay water (24 ppt) was significantly lower than those acclimated in freshwater (0-1 ppt). However, there is an opposite finding where researchers showed that CTmax of threespine stickle back (*Gasterosteus aculeatus*) that acclimated in 20 ppt were significantly higher than those acclimated in 2 ppt (Metzger et al. 2016).

Earlier studies showed that exposing to other stressors beside salinity could also affect CTmax of animals. Three native warm water fishes (*Bidyanus bidyanus*, *Melanotaenia duboulayi*, and *Hypseleotris klunzingeri*) that exposed to organic chemicals such as endosulfan and

chlorpyrifos decreased CT_{max} by 2.5°C (6.1%) and 4.2°C (11.7%), respectively (Patra et al. 2007). LaBlanc et al. (2011) demonstrated in rainbow trout (*Oncorhynchus mykiss*) that fish which chronically exposed to social stress was more susceptible to heat than those remained alone.

Mechanisms of the decline of CT_{max} under salinity stress

The decline of thermal tolerance of tadpoles when acclimated in high salinity is probably due to the energy acquisition and allocation (Sokolova 2013) and/or a dehydration response induced by salinity stress (Everett 2013; Kikawada et al. 2006). When acclimated in high salinity, tadpoles increase the active transport, an energy-expending process, of the branchial Na⁺/K⁺-ATPase (NKA) (Wu et al. 2014) which correlates to the decline of glucose and protein (Gomez-Mestre et al. 2004). The relocation of energy for osmoregulation consequently reduces the ability to resist the heat pressure. In this study, tadpoles were acclimated for seven days before CT_{max} measurement was taken, I contend that the gradual depletion of energy source might weaken the tadpoles to maintain CT_{max} when compared to freshwater tadpoles. Secondly, tadpoles in the high salinity fed less (Floyd 1985; Wu and Kam 2009). Floyd (1985)

reported that tadpoles of *Bufo marinus* which fed with cooked lettuce had significantly higher CTmax than tadpoles treated with starvation in all acclimated temperature (20, 27 and 35 °C, respectively). In this study, I observed tadpoles fed less in high salinity treatments which may lead to starvation. Consequently, starved tadpoles reduce ability to withstand heat pressure.

On the other hand, the decline of CTmax may also relate to the dehydration of animals. Earlier studies showed that exposing to salinity could lead to dehydration in cellular level (Everett 2013; Kikawada et al. 2006). Everatt et al. (2013) reported that the *C. antarcticus* dehydrated during salinity stresses. A similar finding was also found in *F. limnocharis* in that tadpoles gradually dehydrated when they were transferred to a higher salinity treatment (Wu et al. 2014). Earlier studies demonstrated in invertebrate such as *Hodotermes mossambicus* and *Drosophila melanogaster* showed that CTmax could be negatively affected by dehydration (Mitchell et al. 1993; Rezende et al. 2011). Plummer et al. (2003) reported that dehydrated *Terrapene ornata luteola* reduced CTmax by about 2 °C. It has been proposed that cellular dehydration leads to changes in membrane fluidity which might affect

heat tolerance (Everatt et al. 2013). In this study, I contend that dehydration during salinity stress probably reduce tadpoles' ability to withstand the heat pressure.

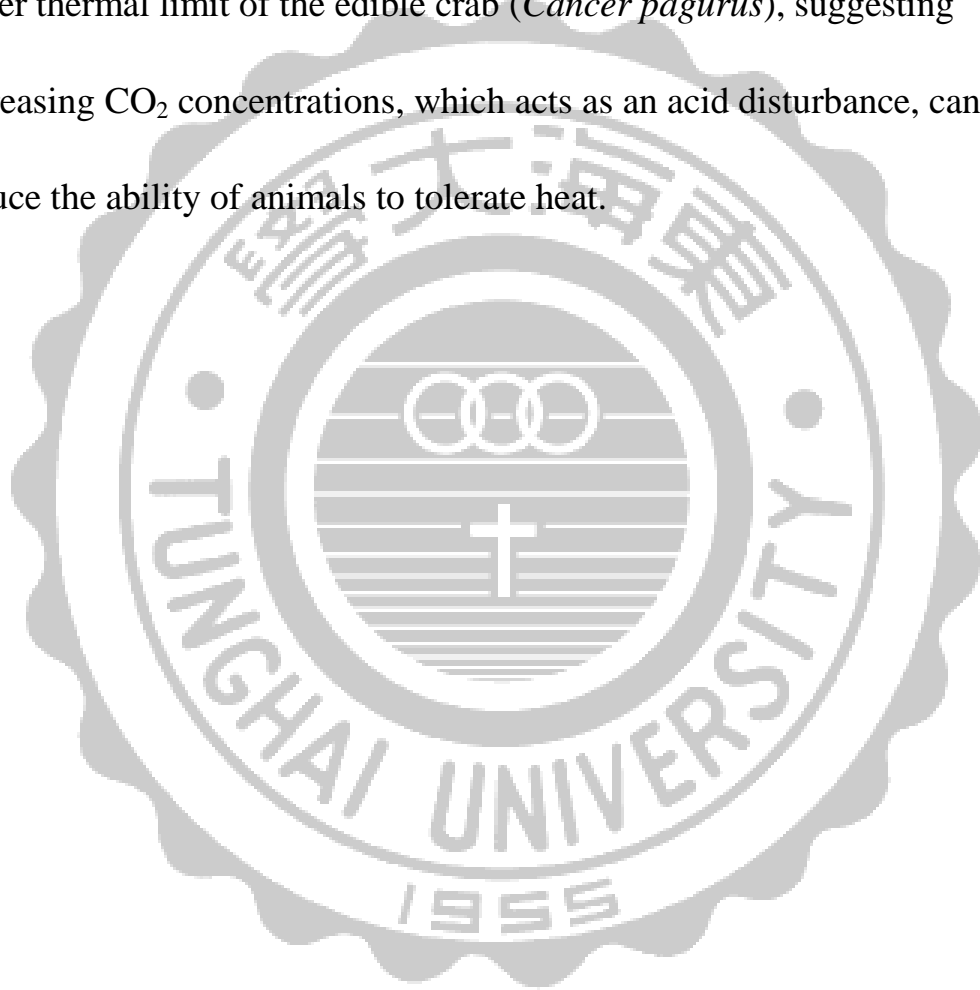
In the case of increased CT_{max} in high salinity in *G. aculeatus* which was associated with the induction of the heat shock proteins (HSPs) expression to improve heat tolerance (Lindquist and Craig 1988; DuBeau et al. 1998; Dahlggaard et al. 1998; Niu et al. 2008). However, the degree of HSPs expression is a function of food availability which relates to protein allocation in different periods of starvation (Koban et al. 1991). The heat shock response can be limited by time due to the body composition changes in responding to nutritional status (Lee et al. 2016), and the regulatory mechanisms which mediates expression of HSPs in a long-term stress exposure may be conserved (Healy et al. 2010). In this study, tadpoles were acclimated for seven days which may cause starvation. Consequently, protein re-allocation in starvation which may depress the expression of HSPs, which can explain why tadpoles had lower CT_{max} in high salinity in the present study.

Conclusion

My finding of the CT_{max} of tadpoles declined in high salinity treatments has important implication on the potential effects of global warming on frog populations at coastal regions. My results suggest that global warming has double impacts on the biology of tadpoles. During the larval period, rising temperature due to global warming on one hand gradually approaches the upper thermal limits of tadpoles which threatens their survivorship (Duarte et al. 2012); on the other hand causes sea level to rise which results in habitat salinization in coastal areas (Day et al. 1995; Feagin and Sherman 2005; Nicholls 2011; Nicholls et al. 2014). The salinity stress not only retards the growth and development but also reduces the ability of tadpoles to tolerate high temperature. Together, global warming negatively impacts the survival and abundance of frogs more than we originally thought, particularly on the frog populations in coastal areas.

The double impacts of global warming may happen in other scenario such as ocean acidification combined with increased temperature on marine animals (Hoegh-Guldberg et al. 2007; Fabry et al. 2008; Kroeker et al. 2013). Accumulation of atmospheric CO₂ has been associated

with rising water temperature and acidification of ocean (Feely et al. 2004; Orr et al. 2005; Doney et al. 2009). The impact of the increasing acidity in aquatic environments may affect the thermal tolerance of animals. Metzger et al. 2007 reported that elevated CO₂ concentrations declined upper thermal limit of the edible crab (*Cancer pagurus*), suggesting increasing CO₂ concentrations, which acts as an acid disturbance, can reduce the ability of animals to tolerate heat.



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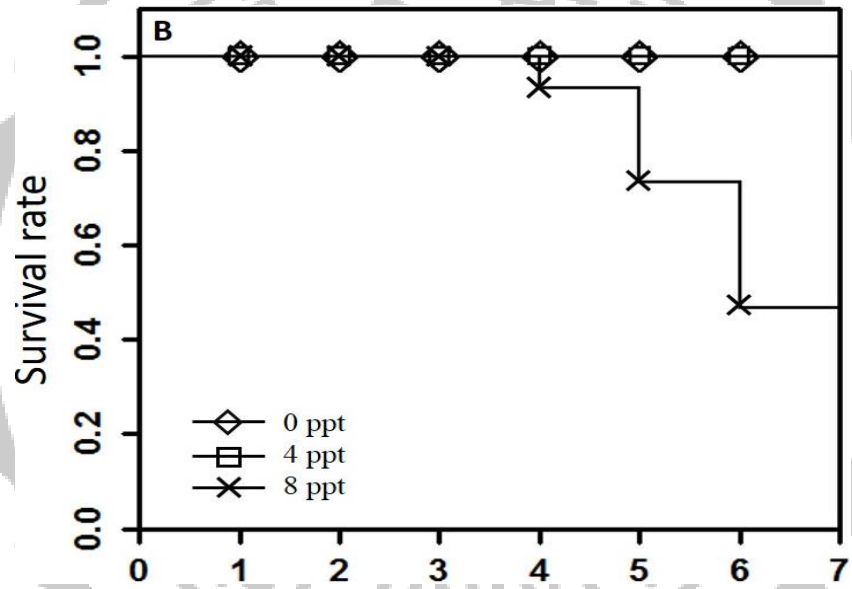
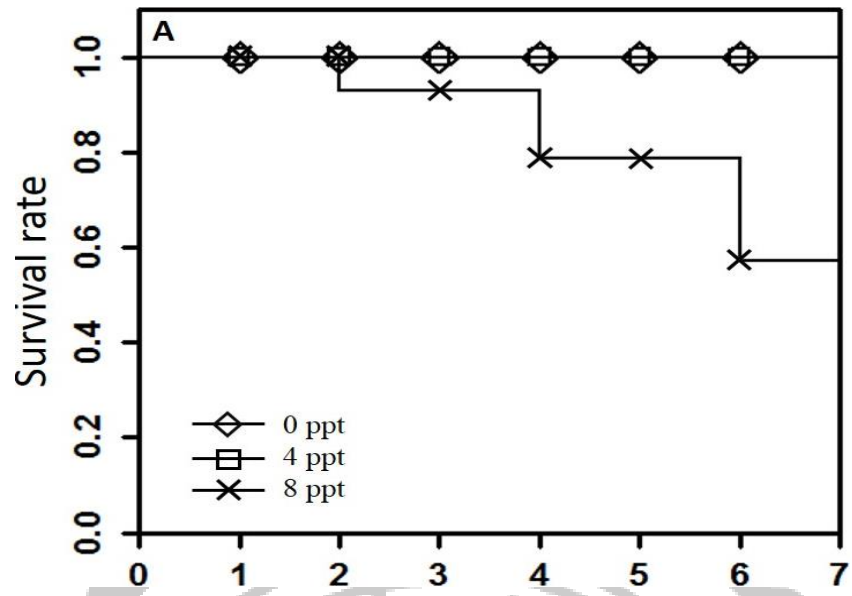
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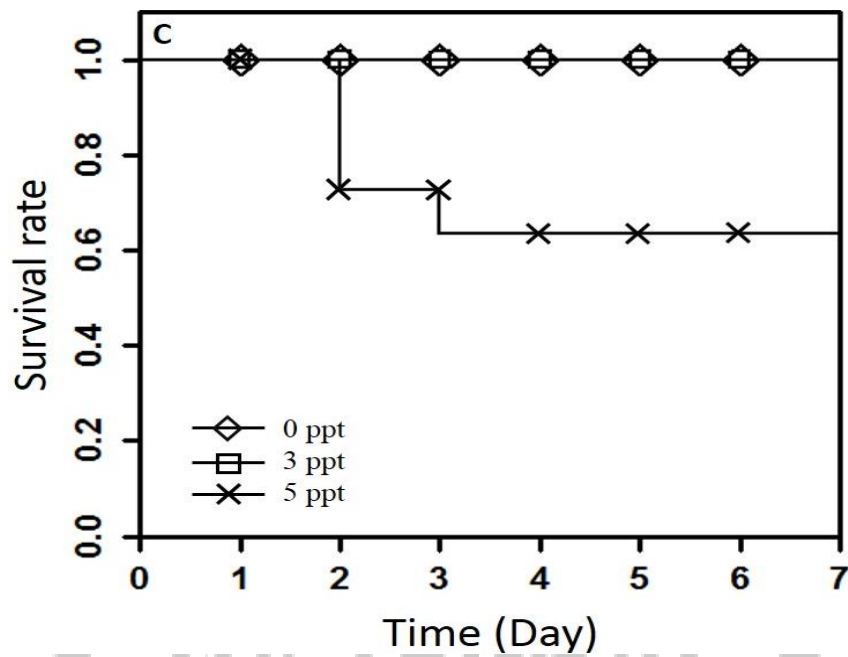
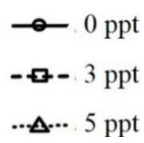
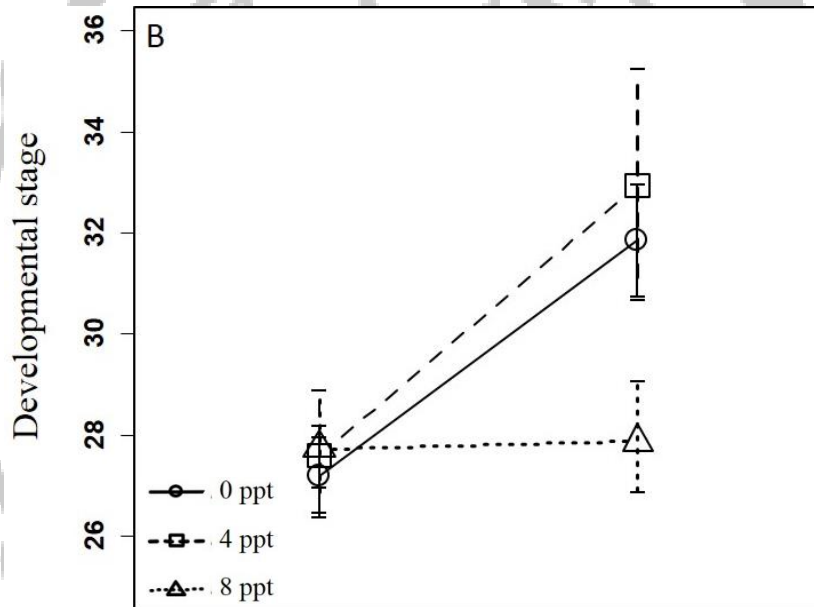
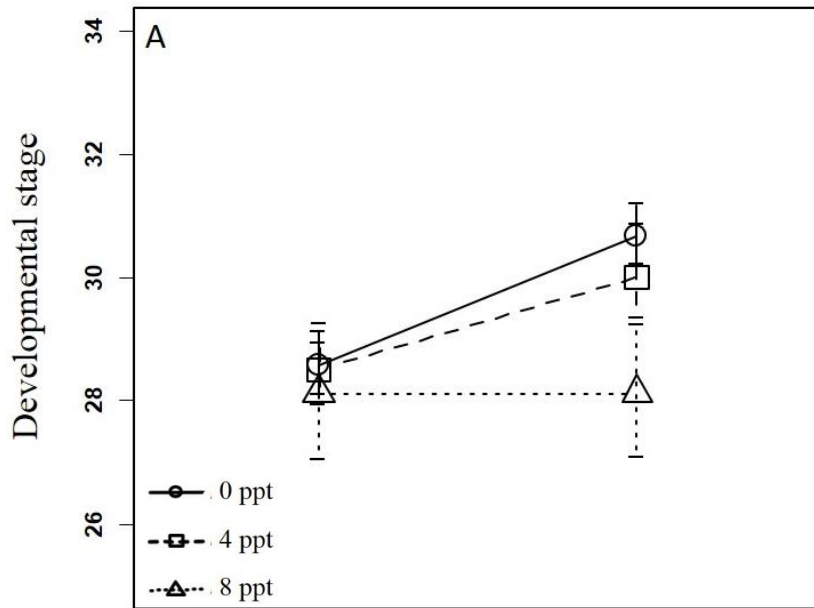


Figure 1. Survival of *Fejervarya limnocharis* (A), *Duttaphrynus melanostictus* (B), and *Microhyla fissipes* (C) tadpoles in freshwater (open rhombus lines), low salinity (open square lines), and high salinity treatments (x-cross lines). *Fejervarya limnocharis*: n=12, 12, and 14, respectively; *Duttaphrynus melanostictus*: n=15, 14, and 15, respectively; *Microhyla fissipes*: n=10, 10, and 11, respectively.



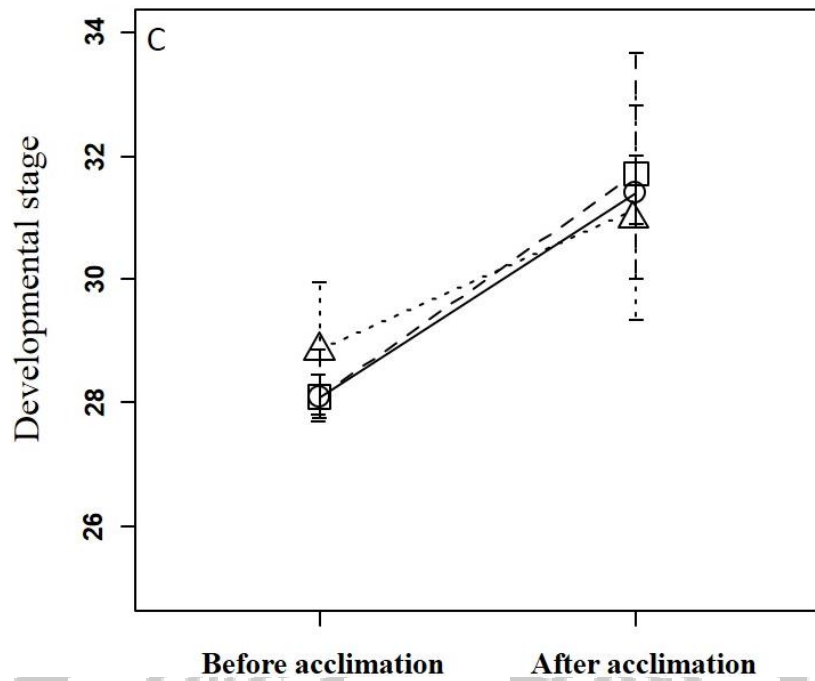
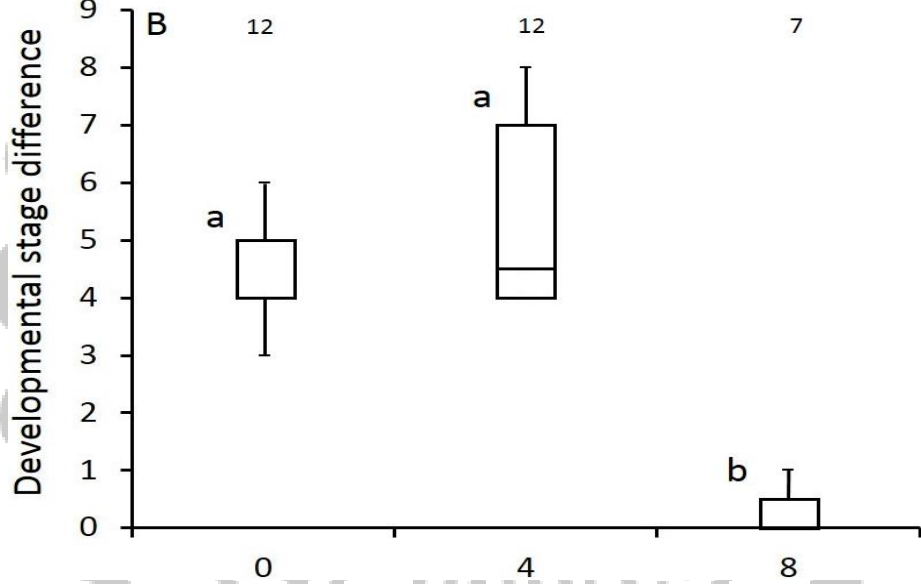
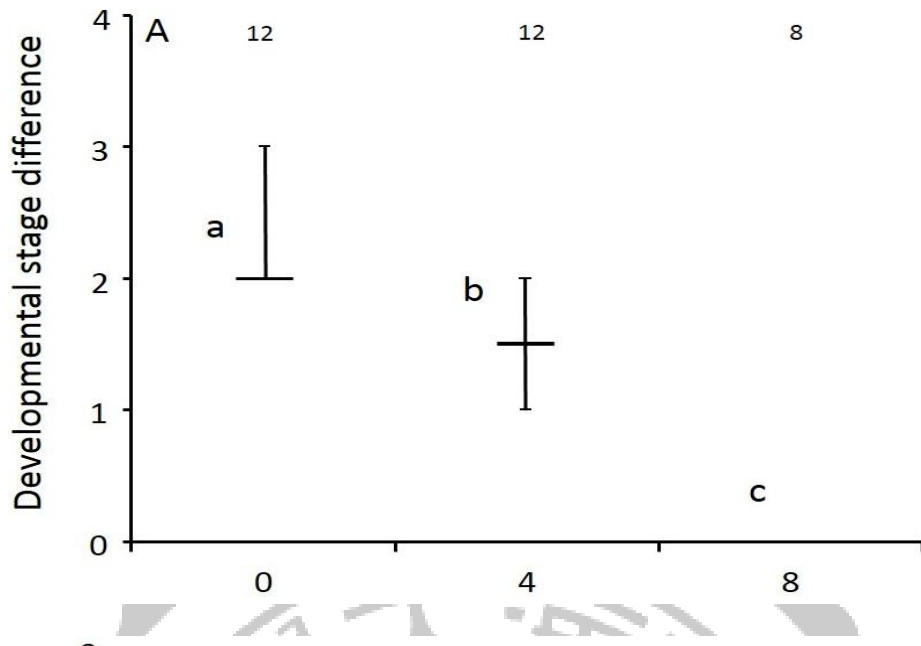


Figure 2. Developmental stage before and after salinity acclimation of *Fejervarya limnocharis* (A), *Duttaphrynus melanostictus* (B), and *Microhyla fissipes* (C) tadpoles in freshwater (open circle), low salinity (open square), and high salinity treatments (open triangle). *Fejervarya limnocharis*: n=12, 12, and 8, respectively; *Duttaphrynus melanostictus*: n=12, 12, and 7, respectively; *Microhyla fissipes*: n=10, 10, and 7, respectively. Data are means \pm SD.



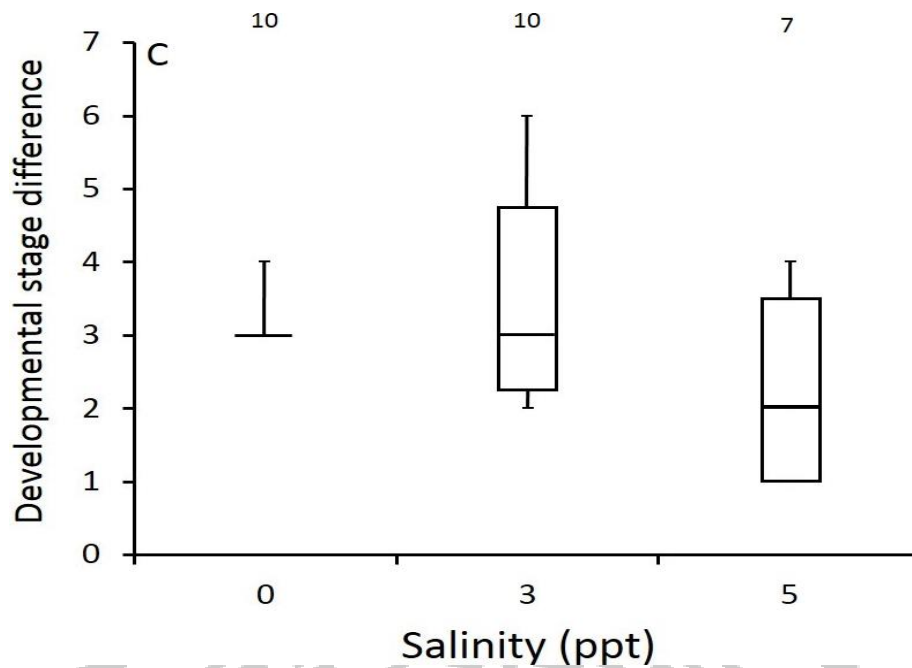


Figure 3. Differences in developmental stage before and after salinity acclimation of *Fejervarya limnocharis* (A), *Duttaphrynus melanostictus* (B), and *Microhyla fissipes* (C) tadpoles in freshwater (0 ppt), low salinity (4, 4, and 3 ppt, respectively), and high salinity (8, 8, and 5 ppt, respectively) treatments. The numbers above the bars are sample size of each treatment. Data are presented as box and whisker plots. The horizontal line indicates the median value. Boxes indicate 25th and 75th percentiles; whiskers indicate minimum and maximum values. Values with different letters represent a statistical difference ($p < 0.05$) among treatments.



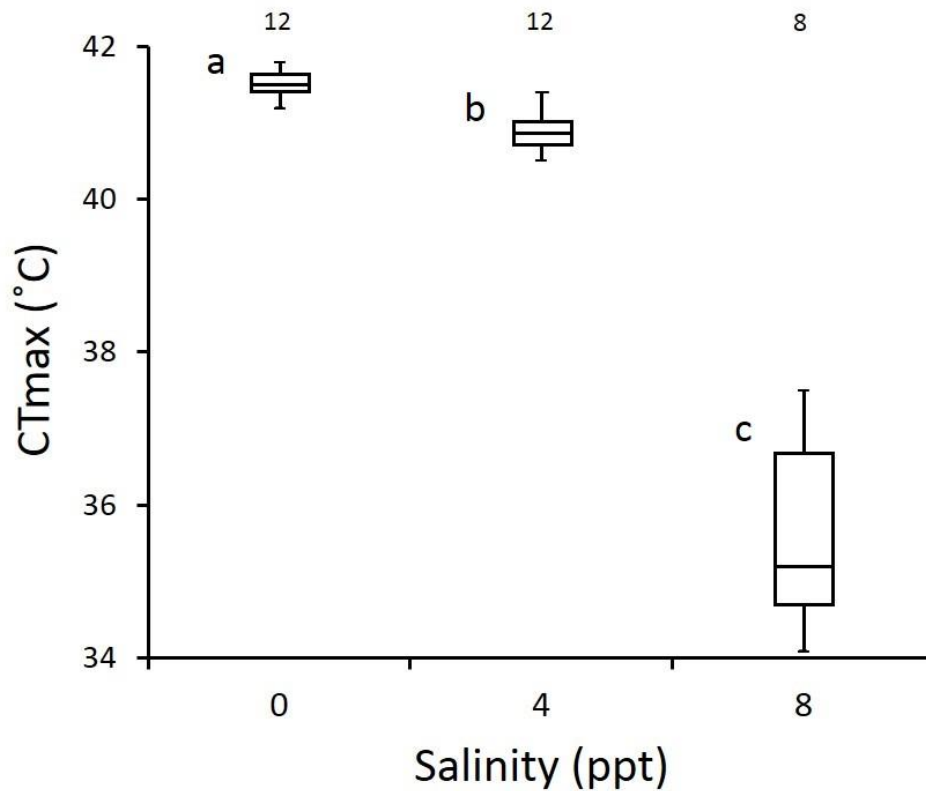


Figure 4. The critical thermal maximum (CTmax) of *Fejervarya limnocharis* tadpoles in freshwater (0 ppt), low salinity (4 ppt), and high salinity (8 ppt) treatments. The numbers above the bars are sample size of each treatment. Data are presented as box and whisker plots. The horizontal line indicates the median value. Boxes indicate 25th and 75th percentiles; whiskers indicate minimum and maximum values. Values with different letters represent a statistical difference ($p < 0.05$) among treatments.

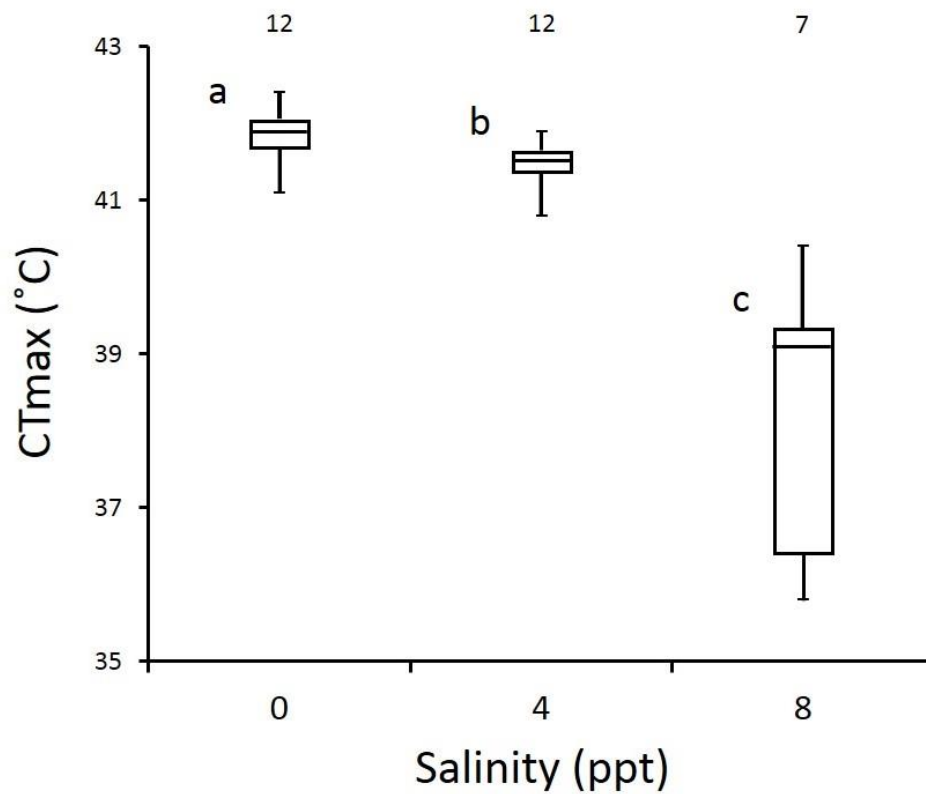


Figure 5. The critical thermal maximum (CT_{max}) of *Duttaphrynus melanostictus* tadpoles in freshwater (0 ppt), low salinity (4 ppt), and high salinity (8 ppt) treatments. The numbers above the bars are sample size of each treatment. Data are presented as box and whisker plots. The horizontal line indicates the median value. Boxes indicate 25th and 75th percentiles; whiskers indicate minimum and maximum values. Values with different letters represent a statistical difference ($p < 0.05$) among treatments.

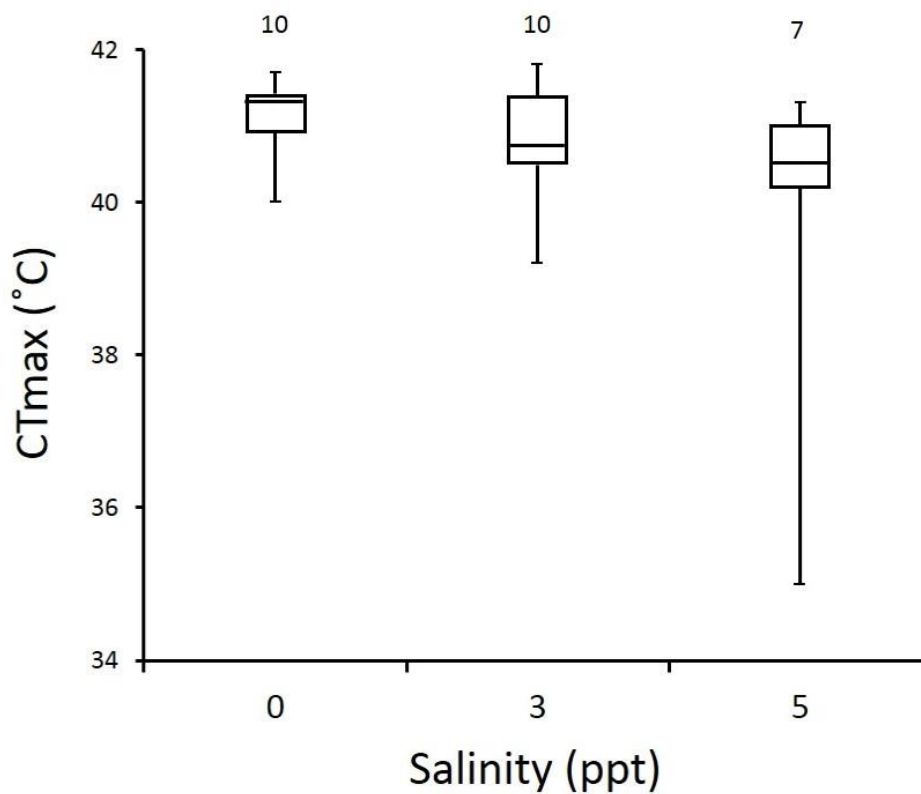


Figure 6. The critical thermal maximum (CTmax) of *Microhyla fissipes* tadpoles in freshwater (0 ppt), low salinity (4 ppt), and high salinity (8 ppt) treatments. The numbers above the bars are sample size of each treatment. Data are presented as box and whisker plots. The horizontal line indicates the median value. Boxes indicate 25th and 75th percentiles; whiskers indicate minimum and maximum values.

Supplemental information

A preliminary test on the salinity tolerance of *M. fissipes*

The aim of this pretest was to measure the salinity tolerance of tadpoles. A total of 50 tadpoles at Gosner stage 26-30 (Gosner 1960) were collected from the windbreak woodlands in Cheng-Xi village of Tainan County and brought back to the laboratory in the summer of 2015. Tadpoles were put into 10 different salinity treatments (1, 2, 3, 4, 5, 6, 7, 8, 9, 10 ppt) for 48 hours (n=5). Each tadpole was kept in a 10.5 × 7.5 × 4.5 cm plastic container filled with 150 ml corresponding solution. The tadpole survivorship was checked every hour in the first 12 hours and then at the 24th and 48th hour.

No mortality was recorded at 1, 2, 3, and 4 ppt at all check points. Contrary, there were two tadpoles died in 5 ppt and three tadpoles died in 6 ppt before 48 hours. Moreover, Tadpoles died within a day when salinity exceeded 7 ppt. Based on the results, I assumed that 6 ppt was the upper salinity limit of *M. fissipes* tadpoles for acclimation experiment. Thus, I set the high salinity treatment at 5 ppt as the sub-lethal concentration and the low salinity at 3 ppt.