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ORIGINAL ARTICLE

The Influence of Oxygen on the Development of *Nanorana parkeri* Tadpoles

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Abstract Ectothermic animals are tolerant of variable oxygen availability, whether low-oxygen levels constrain the fitness of ectotherms remains unclear. *Nanorana parkeri*, an anuran endemic to the southern Tibetan plateau, is an excellent model with which to answer this question. In this study, we raised tadpoles of *N. parkeri* in oxygenated water (high-oxygen group) and deoxygenated unchlorinated tap water (low-oxygen group) and monitored their growth, mortality, and telomere length. The growth rate for body length and body weight was higher in the low-oxygen group than in the high-oxygen group. However, dissolved oxygen did not affect development time, mortality, and telomere length of the tadpoles. These results suggest that although the oxygen concentration influenced some phenotype traits of plateau tadpoles, but it didn't influence the telomere length and survival rate, potential explanations are the local adaptation and *N. parkeri* tadpoles' wide oxygen tolerance, and fluctuant toxic content that resulted in little oxidative stress on tadpoles. These results indicated that low oxygen was not a stress to *N. parkeri* tadpoles' fitness and survival. This study is helpful in understanding the adaptation mechanisms of Tibetan plateau amphibians.

Keywords amphibian, frog, local adaptation, metamorphosis, oxygen concentration, telomere length

1. Introduction

Low temperature, low-oxygen levels, and strong ultraviolet radiation are the main characteristics of the high-altitude environment (Bickler and Buck, 2007; Blumthaler *et al.*, 1997; Scheinfeldt and Tishkoff, 2010). Among these, the influence of low oxygen has received the most attention by ecologists and evolutionary biologists because low oxygen represents a major stress to aerobic metabolism (Gou *et al*., 2014; Wu *et al*., 2013). Some life-history traits, including body size, body mass, growth rate, development time, and mortality determine fitness and survival under given environmental conditions (Semenza, 2000). For example, in a low-oxygen environment, the body size of some ectothermic species (e.g., lizards and frogs) decreases (Cvetković *et al.*, 2009; Liao *et al*., 2010; Ma *et al.*, 2009; Zhang *et al.*, 2012), and the growth rate of a subtropical frog (*Rana nigromaculata*) increases (Liao *et al.*, 2010); the body weight of yaks (*Bos grunniens*) increases (Wang *et al.*, 2006).

In addition, telomere length (TL) represents a promising biomarker of overall physiological state, fitness and of past environmental experiences (Heidinger *et al.*, 2012; Olsson *et al.*, 2011a,b), which could help us understand the drivers of life-history variation in natural populations. A growing number of studies in birds (Pauliny *et al.*, 2006), fish (Debes *et al.*, 2016) and mammals(e.g. roe deer, Wilbourn *et al.*, 2017) suggest that environmental stress or poor environmental

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conditions are associated with shortened TL, but studies of such relationships in ectothermic animals are rare.

Compared with endothermic animals, ectothermic animals are tolerant of variable oxygen availability (Bickler and Buck, 2007). Amphibian hypoxia tolerance is between that of mammals and that of turtles and carp (Bickler and Buck, 2007). For example, ranids can survive a few days of hypoxia at low temperatures (Stewart *et al.*, 2004). Larvae tolerate hypoxia better than adult frogs of the same species (Bradford, 1983; Crowder *et al.*, 1998). Indeed, previous studies have also shown that tadpoles of *Cochranella granulosa*, a lowland species, grew faster and more active in hypoxic water than in oxygenated water (Hoffmann, 2010). However, for plateau ectothermic species, the influence of low oxygen on development remains unknown.

Nanorana parkeri is an anuran endemic to the southern Tibetan plateau and this species has lived in the Tibetan plateau for approximately 19 million years (Che *et al.*, 2010). Because this species occurs across an extensive altitudinal range, which extends from 2850 to 5100 m above sea level (a.s.l.), and has the highest distribution of any amphibian in the world (Hu, 1987), *N. parkeri* is an excellent model with which to study the influence of oxygen levels on fitness-related life history traits and telomere dynamics of plateau ectothermic species. Our question was that whether low oxygen represents a major stress for the development of the Tibetan plateau tadpoles. In this study, *N. parkeri* tadpoles were raised under different oxygen-level conditions until metamorphosis, where upon body size, body weight, development time, and growth rate were recorded, and telomere length was estimated. Based on the performance of lowland amphibians exposed to low-oxygen levels and the local adaptation of plateau species, we hypothesized that low-oxygen levels was not a stressful factor for the development of *N. parkeri* tadpole. Our results will aid in the elucidation of the adaptation mechanisms of the Tibetan plateau amphibians.

2. Materials and Methods

2.1. Egg Collection and Incubation We collected multiple fresh laid egg masses of *Nanorana parkeri* at Bujiu, Nyingtri of Tibet, China (2 939 m a.s.l.) in the middle of May in 2015 and drew experimental population randomly (about 100 eggs) from this mixed egg masses. They were transported to the laboratory at the Institute of Zoology, Chinese Academy of Science, Beijing. In the lab, they were maintained in a climate controlled room at a temperature of 24 ± 0.32 °C and a photoperiod of 12 h: 12 h (L: D). They were raised in standing unchlorinated tap water until the external gills of tadpoles completely disappeared at stage 25 (Gosner, 1960).

2.2. Experimental Treatments To begin the experiment with nearly uniformly sized larvae, tadpole size was measured by Motic Images Plus 2.0 (Motic China Group Co., Ltd.), and 50 tadpoles of nearly uniform size were selected (stage 25, Gosner, 1960) and randomly divided into two groups. In the low-oxygen group (LOG), 30 tadpoles were maintained in deoxygenated, unchlorinated tap water with nitrogen injection. In the high-oxygen group (HOG), 20 tadpoles were raised in water oxygenated with an aquarium pump. A special facility was developed, which consisted of two tanks, one with bubbling nitrogen, and the other with bubbling oxygen. Thirty (radius = 7.5 cm, height = 10 cm) and 20 bowls were placed in the nitrogenated and oxygenated tank, respectively. To allow water to circulate, each bowl had numerous pores (radius = 0.05 cm) on the bottom and sides, and each tadpole was raised in an independent bowl. The bowls in the LOG were covered with plastic lids to maintain the low oxygen levels. The temperature for the two groups was 17 ± 0.24 °C, which was set according to the average temperature in the field in the high-altitude region of the Tibetan plateau in May and June (Basang, 2005). The photoperiod was 12 h: 12 h (L: D). Each tadpole was fed chopped spinach (0.03 g) twice a week in conjunction with water changes. Eighty percent of the water was changed twice a week for both groups, when the water was changed, feces and excess food were removed by siphoning. Because dissolved oxygen (DO) ranged from 4.6 ± 0.02 mg/L to 5.4 ± 0.01 mg/L at 4,300 m a.s.l., and ranged from 5.5 ± 0.03 mg/L to 11 ± 0.02 mg/L at 2 850 m a.s.l. in the field (Fan, pers. obs.), in our experiment, DO was set at 5.00 ± 0.30 mg/L in the LOG, and 8.25 ± 0.20 mg/L in the HOG, which was the average DO concentration in field at 4,300 m a.s.l. and 2 850 m a.s.l., respectively. The DO concentration was measured with a YSI-55 DO meter (YSI, Yellow Springs, Ohio, USA) three times daily. Nitrite concentration was assessed using the colorimeter HACH DR/850 (Hach Company, Loveland, Colorado, USA) before each water change. We also measured the value in the field at the corresponding HOG and LOG habitat at 2 850 m a.s.l. and 4 300 m a.s.l., respectively.

When the tadpoles were approaching metamorphosis (stage 42; occurrence of at least one forelimb; Gosner, 1960), the bowls were checked twice a day to determine the timing of metamorphosis (i.e., development time =

days elapsed from reaching Gosner stage 25 to reaching stage 46). At the end of metamorphosis (stage 46, tail completely resorbed, metamorphosis complete), fresh body mass and snout-vent length (SVL, the length from the tip of snout to the end of vent) were measured with an electronic balance (to the nearest 0.0001 g; Mettler-Toledo GmbH, Greifensee, Switzerland) and digital Vernier calipers (to the nearest 0.01 mm; Kanon Instruments, Japan), respectively. The average growth rate (g/d) for body weight for each individual was estimated by dividing body weight at metamorphosis by development time. The average growth rate for body length was also estimated using body length increments (mm/d), similar to that of body weight. Mortality was also recorded. Metamorphosed tadpoles were euthanized with an overdose of MS-222, and heart, liver, and muscle were harvested and frozen at –80 °C. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences (Permit Number: IOZ11012).

2.3 DNA Isolation and Quantitative PCR Genomic DNA was isolated from the heart, liver, and muscle using a DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. DNA quantification was performed using an ND-1000-Spectrophotometer (NanoDrop, Wilmington, Delaware, USA).

Telomere length was quantified using real-time quantitative PCR developed to measure relative telomere length, as previously described in Cawthon (2002) and Criscuolo *et al.* (2009). The relative telomere length was expressed as a ratio of telomere repeat copy number (T) to a control single-gene copy number (S) (Cawthon, 2002). Quantitative PCR was performed using the SYBR Select Master Mix (Takara, Dalian, China) with an ABI PRISM 7500 (Applied Biosystems, Foster City, California, USA). The forward telomere primer was 5′-CGGTTTGTTTG GGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′, the reverse telomere primer was 5′-GGCTTGCCTTACCCT TACCCTTACCCTTACCCTTACCCT-3′ (Callicott and Womack, 2006). Because the 18S rRNA gene is highly shared between humans and frogs (Mallatt and Winchell, 2007), we used 18S as the non-variable copy number gene with the following primer sequences originally designed for humans (Takara Bio Inc., Shiga, Japan): forward primer, 5′-ACTCAACACGGGAAACCTCA-3′, reverse primer 5′-AACCAGACAAATCGCTCCAC-3′. QPCR for both 18S and telomeres was performed using 25 ng of DNA per reaction. The total volume was 10 μL (9 μL of master mix + 1 μL of DNA). The master mix contained 2 μL of each primer and 5 μL of Applied Biosystems SYBR Green PCR master mix. PCR conditions for telomeres and 18S were 5 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 60 °C, and 15 s at 72 °C. Both reactions ended with a dissociation program of 1 min at 95 °C, 30 s at 55 °C, and 30 s at 95 °C.

Telomere length was expressed relative to the single-copy number gene (18S) measured using the same sample of DNA. The telomeric DNA relative to the constant 18S amplicon was calculated with the following formula: telomere length = $2^{(-\Delta Ct)}$ where $\Delta C_t = C_t^{\text{Telomere}} - C_t^{18S}$ (Cawthon *et al*., 2002).

2.4 Statistical analysis We tested the normality of distributions and homogeneity of variances for the data with the Kolmogorov-Smirnov and Levene's test, respectively, prior to analysis. Group means for body weight, body length, growth rate for body weight, growth rate for body length, development time, nitrite concentration, and telomere length were compared using an independent-sample *t*-test. Results were presented as means ± SE per group. A value of $P < 0.05$ was considered statistically significant. Differences in mortality between the two groups were compared using Fisher's exact test. All analyses were performed using SPSS ver. 17.0 IBM software (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. Fitness-related traits Body length ($df = 14$, $t = –3.936$, $P = 0.001$, Figure 1A), body weight ($df = 14$, $t = –4.101$, $P = 0.001$, Figure 1B), growth rate for body length ($df = 25$, $t = –3.038$, $P = 0.006$), and body weight ($df = 25$, $t = –4.782$, $P = 0.002$) were greater in the LOG than the HOG. As a result, the tadpoles grew to a larger size in terms of body length and weight at metamorphosis (Figure 1A, B). However, DO did not affect the development time ($df = 14$, $t = –0.588$, $P = 0.566$, Figure 1C) or mortality of tadpoles (60% *vs.* 84%, $P = 0.100$).

3.2. Nitrite concentration The nitrite concentration of the HOG and LOG ranged from 0 to 1.152 ± 0.0002 mg/L and from 0 to 0.516 ± 0.0005 mg/L, respectively. The mean value of the nitrite concentration in the HOG was significantly higher than that in the LOG (0.096 ±.0.0003 mg/L *vs.* 0.043 ± 0.0002 mg/L, $df = 25$, $t = 137.597$, $P < 0.001$).

3.3. Telomere length Telomere length did not differ between the two groups of tadpoles in the heart ($df = 30$, $t = –1.599$, $P = 0.138$, Figure 2A), liver ($df = 30$, $t =$

–1.054, $P = 0.313$, Figure 2B), or muscle ($df = 30$, $t = -1.419$, $P = 0.183$, Figure 2C).

4. Discussions

4.1. Oxygen Level and Fitness-Related Life History Traits Our results showed that oxygenated water was not beneficial to the development of tadpoles; on the contrary, to some extent, the low oxygen concentration was more beneficial to tadpole development. This was consistent with the findings for a lowland species, *Cochranella granulosa* (Hoffmann, 2010). This can be attributed to the concentration of various nitrogen compounds derived from tadpole feces in hypoxic and oxygenated water, in

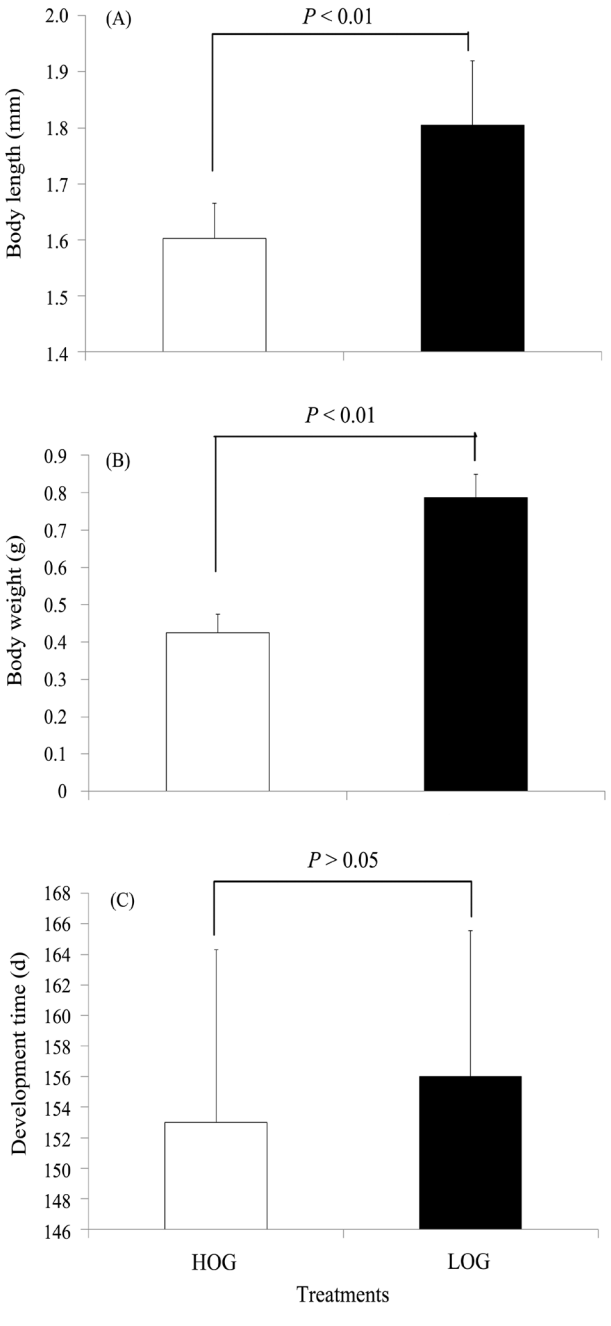

Figure 1 The group means for body length (A), body weight (B), and development time (C) when metamorphosis was completed in the low-oxygen group (LOG) and the high-oxygen group (HOG) as compared by an independent-sample *t*-test; values are means ± SE.

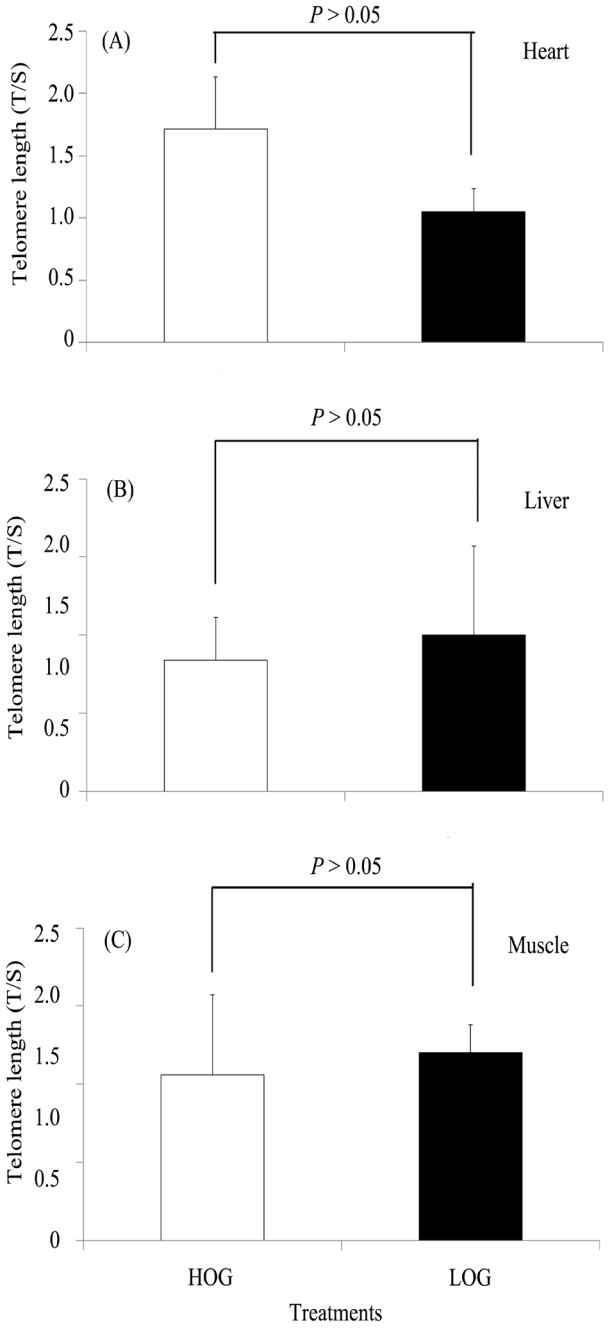

Figure 2 The group means of relative telomere length of *Nanorana parkeri* in heart (A), liver (B), and muscle (C) in the low-oxygen group (LOG) and high-oxygen group (HOG), as compared by an independent-sample *t*-test; values are means ± SE.

oxygenated water, ammonium was oxidized to nitrates, and nitrates were secondarily reduced to nitrites. Nitrites are toxic, and as nitrites pass through the bloodstream, they transform hemoglobin into methemoglobin, which causes methemoglobinemia (Hoffmann, 2010). The mean nitrite concentrations of the HOG and LOG groups were similar to the means at low (2 850 m a.s.l.; 0.105 ± 0.0015 mg/L) and high altitude (4 300 m a.s.l.; 0.046 ± 0.0012 mg/L) in the field, respectively. Therefore, LOG also experienced a low nitrate level, which was beneficial to tadpole development.

4.2. Oxygen Level and Telomere Length Dynamics

Based on the results of life-history traits, we hypothesized that the HOG should have shorter telomere than the LOG because the toxic nitrites induced oxidative stress (Ferrario *et al.*, 2009), and oxidative stress causes an increased number of single-strand breaks leading to loss of distal telomere fragments and accelerated telomere shortening (von Zglinicki *et al.*, 2002). For example, telomere length was shortened when sand lizards (*Lacerta agilis*) were exposed to a stressful environment (Olsson *et al.*, 2010). However, we didn't observe significant difference in telomere length between these two groups. Two possible factors attributed to this result. First, although the body length, body weight and the growth rate was significant greater in LOG than in HOG, the oxygen levels were set based on corresponding field altitude conditions, due to local adaptation and wide oxygen-level tolerance, no matter the oxygen concentration or nitrite concentrations, neither of them was not stress to change telomere length. Secondly, although toxic sources led to paler heads for tadpoles in the HOG than in the LOG, and the toxic water delayed the growth of tadpoles in the HOG, the nitrate content fluctuated because the water was changed twice a week. Thus, the harmful environment was not a consistent and long-lasting stress that could accelerate telomere shortening. Indeed, the nitrate content also fluctuated in field for the frequent rainfalls. Therefore, there was no significant difference in telomere length between the two groups.

In conclusion, although the oxygen concentration influenced some phenotype traits of plateau tadpoles, but it didn't influence the telomere length and survival rate, which indicated that low oxygen was not a stress to *Nanorana parkeri* tadpoles' fitness and survival. This is the first exploration of the influence of oxygen level on the telomere length of a native Tibetan plateau amphibian. Our study provided new insights for telomere assays in ecology and evolution, and further studies on the adaptive significance of these effects are warranted in the future.

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