**ORIGINAL ARTICLE**

# The Expression Plasticity of Hypoxia Related Genes in High-Altitude and Plains *Nanorana parkeri* Populations

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**Abstract**    For species that have a broad geographic distribution, adaptive variation may be attributable to gene expression plasticity. *Nanorana parkeri* is an anuran endemic to the southern Tibetan Plateau where it has an extensive altitudinal range (2850 to 5100 m asl). Low oxygen concentration is one of the main environmental characteristics of the Tibetan Plateau. Hypoxia-inducible factor α subunits (HIF-1α and HIF-2α, encoded by Endothelial PAS domain protein 1 (*EPAS1*)) and associated genes (e.g., vascular endothelial growth factor (*VEGF*) and Erythropoietin (*EPO*)) play crucial roles in maintaining oxygen homeostasis. In this study, we compared the expression of *HIF-1A*, *VEGF*, *EPAS1* and *EPO* mRNA between two populations of *N. parkeri*: one population inhabiting the native high altitudes, and the second living in, and being acclimated to, the lower plains (70 m asl). The expression of *HIF-1A*, *VEGF* and *EPAS1* mRNA in the high altitude population were significantly higher than in the acclimated population, whereas there was no significant difference for *EPO* between two groups. Our results indicated that gene expression plasticity may make significant contributions to local adaptation of species that have broad altitudinal distributions. In addition, we deepen our understanding of the adaptive potential of this species by evaluating the experiments in the scope of its evolutionary history.

**Keywords**  local adaptation, ectotherms, de-adaptation, plasticity, Qinghai-Tibetan plateau, amphibians

## 1. Introduction

Understanding population responses to rapid environmental changes is a research frontier. Organisms can adapt to environmental variation by phenotypic plasticity. Phenotypic plasticity is defined as the ability of a given genotype to produce different phenotypes in response to different environmental conditions (Agrawal, 2001). It is a common phenomenon and occurs in all organisms from microbes to mammals. Exploring the genetic basis of phenotypic plasticity will help us to understand the adaptation and evolutionary responses of organisms. In theory, plasticity may be caused by up- or down-regulation of common developmental pathways, but also by environment-specific gene expression (Snell-Rood *et al*., 2011). The patterns associated with gene expression changes in plasticity might deepen our understanding of the evolution of stress resistance (DeWitt *et al*., 1998).

For example, hypoxia is one of the main environment characteristics of a mountain range's plateau area. Hypoxic condition is hostile to the heart because it affects

cardiac function severely (Zhou *et al.*, 2012). Native organisms with long-term adaptation to high altitude environments have evolved a set of specific phenotypic traits to survive this harsh environment. For example, the hypoxia-inducible factor (HIF) pathway is induced by the hypoxic environment and it can protect the organisms from detrimental effects of hypoxia. HIFs are composed of a labile hypoxia-regulated α subunit, so called HIF-1α, -2α or -3α, and a constitutive β subunit (Wenger and Gassmann, 1997). The HIF α subunits are functional parts, mediating many genes involved in erythropoiesis, angiogenesis, autophagy, and energy metabolism (William and Peter, 2008). HIF-1α and -2α mediate several genes, for example, transcription of vascular endothelial growth factor (*VEGF*) (Forsythe *et al.*, 1996), which is a major mediator of vasculogenesis and angiogenesis and protects endothelial cells from undergoing apoptosis (Nor *et al.*, 1999); HIF-2α is encoded by *EPAS1*, which mediates hypoxia-induced expression of the erythropoietin (*EPO*) gene (Kapitsinou *et al.*, 2010). *VEGF* and *EPO* are of particular importance because they play major roles in promoting $O_2$ delivery and metabolic adaptation to $O_2$ deprivation in mammals (Ahmad *et al.*, 2013; Bigham *et al.*, 2010; Espinoza *et al.*, 2014; Li *et al.*, 2009, 2013; Nor *et al.*, 1999; Simonson *et al.*, 2012; Wang *et al.*, 2006; Yi *et al.*, 2010; Zhao *et al.*, 2004).

However, recent studies have shown that when organisms native to high altitudes were exposed to sea level oxygen conditions, 50%–80% ( Gao, 2005) showed a series of clinical symptoms including dizziness, palpitation, hypersomnia, malaise, chest tightening, precordial ache, arrhythmia, hypophrenia; some show abnormal readings on physiological parameters for heart, lung and blood, which continues to drop even after reaching normal plain level, i.e., overcompensated; a few individuals still present symptoms including hypoproteinemia, oligocardia, decreased cardiac function and pulmonary hypertension after two years of continuous stay in the plains; in some cases, this may last for a few years, and in the most severe cases the individuals were forced to return to high altitude (Gao, 2005; Zhang *et al.*, 1996). We refer to all the above pathological features as symptoms of "high altitude de-adaptation reaction" or "high altitude de-adaptation syndrome" (Gao, 2005; Zhang *et al.*, 1996). High altitude acclimation and adaptation mechanisms have been well described, however, high altitude de-adaptation mechanisms are studied more rarely.

Compared with endothermic animals (e.g., most

bird and mammals), amphibians are tolerant of variable oxygen availability. For example, a frog's anoxia tolerance is in between the anoxia sensitivity of mammals and anoxia tolerance of turtles and carp. Ranids survive a few days of anoxia at low temperatures and approximately 3 h of anoxia at room temperature (Stewart *et al.* 2004).

*Nanorana parkeri* is an anuran endemic to the southern Tibetan plateau, which is the highest plateau in the world (at greater than 4000 m a.s.l.); and low atmospheric oxygen pressure (about 40% lower than at sea level) is one of its main environmental characteristics. *N. parkeri* distributes across a narrow latitudinal (28 to 31°N) but extensive altitudinal range (2850 to 5100 m a.s.l.).

In this study, to further understand de-adaptation of frogs, we transferred the high altitude *N. parkeri* population to the plains (only 70 m a.s.l.), and compared the expression of four crucial genes of the HIF pathway (*HIF-1A*, *VEGF*, *EPAS1* and *EPO*) in *N. parkeri* in heart tissues between a high altitude native population and the population housed at only 70 m a.s.l. in the laboratory in Beijing. More specifically, we asked (1) whether *N. parkeri* will be subjected to a high altitude de-adaptation reaction when they are transferred to the plain? (2) What is the genetic mechanism involved in *N. parkeri* phenotypic plasticity (local adaptation) in the plain?

## 2. Materials and Methods

**2.1 Sample preparation**   One high altitude native *N. parkeri* population (six males and five females) was sampled from Mainling (29.31°N, 94.37°E; 4312 m a.s.l.), in Nyingtri county, Tibet, in June 2014 (Table 1). Animals were killed by breaking their spine immediately upon capture to harvest the heart. Tissues preserved in RNA holder (TransGen Biotech Co., Ltd., Beijing, stored at room temperature), were brought to our laboratory in Beijing and used for RNA extraction.

Another population (12 males and 12 females) was sampled from Damshung (30.27°N, 91.04°E; 4500 m a.s.l.), Lhasa, Tibet in May 2013. The population was transferred to the lab in Beijing (Institute of Zoology, Chinese Academy of Sciences, Beijing; 70 m a.s.l.) and raised in the lab. Eight frogs were raised together in three 65×35×40 cm³ plastic boxes, whereby in each box four males were housed with four females. The density is similar to that of some wild population in breeding season at high altitude (pers. comm. Liqing Fan, associate professor in College of Agricultural and Animal Husbandry, Tibet University). The boxes were placed outside in a garden area. Window nets covered the boxes

**Table 1** Samples information.

| Sampling time [year.month] | Location | Coordinates | Altitude a.s.l | Treatment |
|---|---|---|---|---|
| 2014.6 | Mainling, Nyingtri, Tibet | 29.31°N, 94.37°E | 4312 m | Killed on spot |
| 2013.5 | Damshung,Lhasa, Tibet | 30.27°N, 91.04°E | 4500 m | Acclimated in lab in Beijing (70 m), killed in 2014.6 |

to ensure ventilation. In order to imitate the frogs' natural habitat, grass, mud and shelter were placed in each box (Figure 1), and the water in each box was replaced every three days. We fed crickets and mealworms to the frogs once a day, the amount of food given to each frog was eight 1.5 cm long crickets and ten 1.5 cm long mealworms. At the end of October of 2013, hibernation began and lasted until March 2014. During hibernation, 10 cm of soil layer was added to cover the bottom of each box, and 5 cm water layer was above the soil layer. The frogs stayed in the bottom of the soil layer, then the boxes were put in the incubator under 6°C (Li, 2014). Water levels were replenished every two weeks to avoid water loss. The acclimation lasted for one year. RNA extraction from the same tissue and with the same methods as for the high altitude native population took place in June of 2014. During the raising process, we recorded the exact time of starting to accept food, the first embracing behavior occurrence and going into hibernation of each frog.

All procedures involved in the handling and care of animals were in accordance with the China Practice for the Care and Use of Laboratory Animals and were approved by China Zoological Society.

**2.2 RNA extraction and primer preparation** Total RNA was extracted and purified from *N. parkeri* heart using TRIZOL reagent (Invitrogen). The concentrations of RNA samples were quantified with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., DE/ USA) for further analyses.

We obtained *HIF-1A*, *VEGF*, *EPAS1* and *EPO* gene sequences according to the whole-genome sequence of *N. parkeri* (Sun *et al.*, 2014) and homologous sequences of Human (*Homo sapiens*), yak (*Bos grunniens*), common frog (*Rana temproraria*), rainbow trout (*Oncorhynchus mykiss*), African clawed frog (*Xenopus laevis*) and tropicalis frog (*Xenopus tropicalis*) from GenBank (Benson *et al.*, 2011). The designed PCR primers are shown in Table 2. All of the primers were produced by Shanghai Biotechnology Corporation (Shanghai, China).

**2.3 RT-PCR** Reverse-transcription polymerase chain reaction (RT-PCR) was performed with the Access RT-PCR System (Promega) according to the manual. A total of 0.6 µg RNA isolated from *N. parkeri* heart for each population from each of five individuals were pooled into a total aliquot of 3 µg and reverse transcribed for 60 min at 42°C and for 10 min at 75°C with M-MLV reverse transcriptase. RT-PCRs were performed by using SYBR green PCR Master Mix (Applied Biosystems) in a 10 µl total volume, including 5 µl premix, 2 µl 1 µM each primer and 1 µl cDNA template to quantify the expression of *HIF-1A*, *VEGF*, *EPAS1* and *EPO* mRNA. The amplification was performed for 40 cycles at the
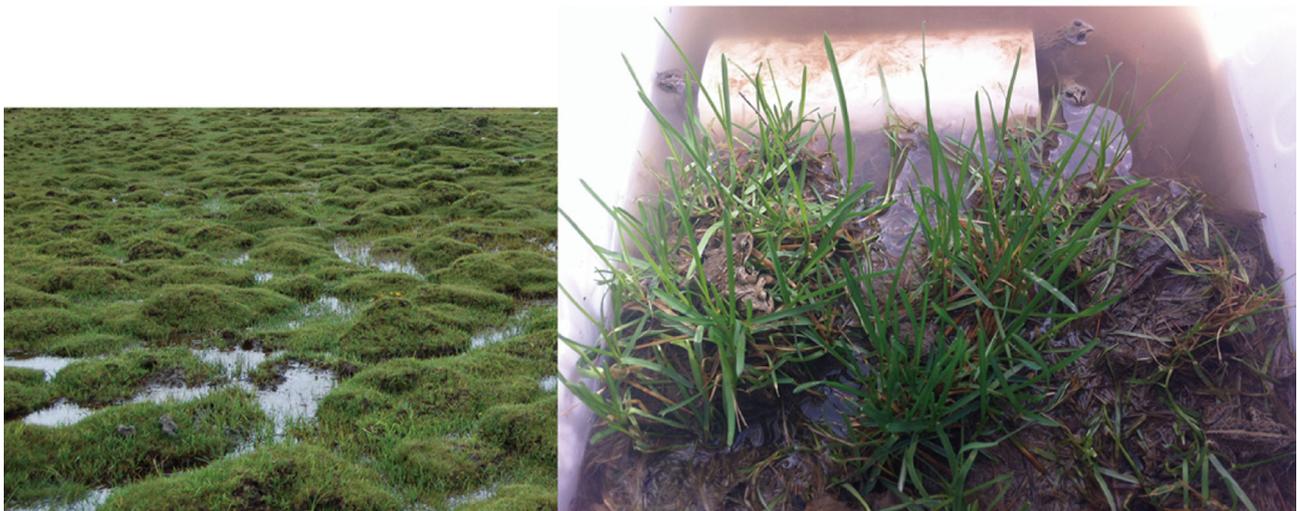


**Figure 1** *Nanorana parkeri* habitat. A. natural habitat in Tibet; B. artificial habitat in laboratory in Beijing.

following cycle conditions: 95°C for 10 s (denaturation), 56°C for 10 s (annealing) and 72°C for 20 s (extension). Each reaction was performed in triplicate. To compare among groups, mRNA levels of target genes were measured as relative expression using $2^{-\triangle\triangle CT}$ values and normalized to $\beta$-Actin generated from the same sample (Livak and Schmittgen, 2001).

**2.4 Statistical analysis of data** Results were presented as mean ± S.E. per group. The mean derived from three replicates, but not from the five individuals, because the tissues from five individuals were mixed as one sample. Group means were compared by an independent-sample $t$ test. A value of $P < 0.05$ was considered statistically significant (SPSS ver. 17.0).
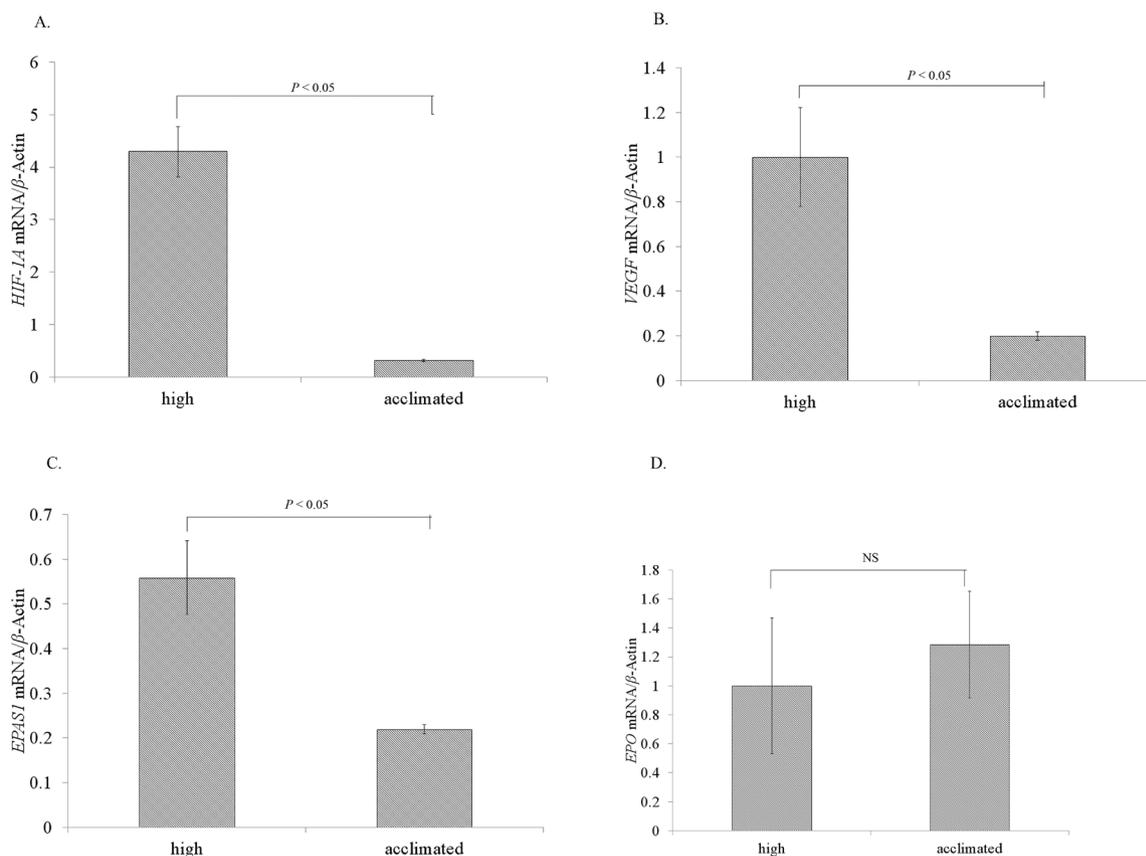
# 3. Results

**3.1 Behavioral observation of the acclimated population** The frogs immediately started with food intake when they were transferred to the laboratory in Beijing. During the natural breeding season, the

**Table 2** Primer details for RT-PCR.

| Primer name | Primer sequence (5'-3') |
| --- | --- |
| β – actin-F | CTCTGCGTCTTGACTTGG |
| β – actin-R | GCTGTAGCCATTTCTTGC |
| HIF-1α-F | ACCCAACAAACCCCGCG |
| HIF-1α-R | GATCGAGGGCTCTTAATAA |
| EPAS1-F | TCTACAATACACGCAACTC |
| EPAS1-R | TGGTTCCTCCTTCAGC |
| VEGF-F | TATCAAAGTCGCAAACC |
| VEGF-R | TATCCCACTGCCAACC |
| EPO-F | ACGCAGAATGGAGGAAGC |
| EPO-R | CCTGTAAACACCGTAGAAT |

**Table 3** Behavioral performance of the acclimated *N. parkeri* population in the laboratory in Beijing.

| Behavior | Time |
| --- | --- |
| Start to accept food | Immediately when they were transferred to the laboratory |
| Mating behavior | The third day after they were transferred to the laboratory |
| Hibernation | At the beginning of October |



**Figure 2** Expression of each gene's mRNA in the high altitude native group and the acclimated group. Expression levels were normalized to *β-actin* mRNA levels. Representative results from three independent experiments in triplicate on the same mRNA of different individuals are presented as means ± standard errors. (A) *HIF-1A*; (B) *VEGF*; (C) *EPAS1*; (D) *EPO*.

laboratory frogs engaged in typical breeding behavior frequently, but did not produce eggs, eventually. The first embracing behavior was observed on the third day after they were transferred to the lab. At the beginning of October, the laboratory frogs went into hibernation gradually. This happened at a similar time compared to the wild (Table 3).

**3.2 mRNA expression of the acclimated population in comparison with the high altitude native population**
The gene expression levels of *HIF-1A*, *VEGF* and *EPAS1* mRNA of the high altitude native population were significantly higher than in the acclimated population (*P* < 0.05, Figure 2A, B and C, Table 4), but not the levels of *EPO* that did not differ significantly between the high altitude group and the acclimated group (Figure 2D).

## 4. Discussion

In our study, we observed potential gene expression acclimation of *HIF-1A*, *VEGF* and *EPAS1*, but not for *EPO*. It seems that the *EPO* response to change of environment is either absent, or not very pronounced. A similar result was found in previous studies (Robach *et al*., 2004); such as when the hypoxic stimulus persists, EPO increased during the first days (about 1 week), then progressively declined. In our study, we find no significant EPO response and the reason for a lack of an EPO response in this case still remains unclear (Robach *et al*., 2004).

Overall, we here report on the general lack of the high altitude de-adaptation response. These results indicate that the frogs successfully adapted their metabolism to the sea-level altitude environment. A similar study on deer mice (*Peromyscus maniculatus*) which distribute from highland (4350 m) to lowland (430 m) areas elucidated the role of regulatory plasticity in evolutionary adaptation

to hypoxic cold-stress (Cheviron *et al*. 2013). Another study of a broadly distributed Andean bird species (*Zonotrichia capensis*) also documented a large degree of transcriptomic plasticity in skeletal muscle in response to changes in elevation (Cheviron *et al*., 2008). Furthermore, Cheviron *et al*. (2012) suggested that the elevated thermogenic capacities of highland mice were largely attributable to changes in gene expression plasticity. Together, these studies suggest that regulatory plasticity, and not longer-term natural selection for specific alleles at the actual genes, may make significant contributions to the niche breadth of species that have broad altitudinal distributions.

In addition, the evolutionary history of *N. parkeri* illustrates the adaptation potential of this species to the lowland environment. *N. parkeri* belongs to the subgenus *Nanorana*. The split of the subgenera *Nanorana* and *Paa* took place about 9 Mya, which is compatible with the geological hypothesis that the rapid uplift of Tibet took place about 8 Mya (Harrison *et al*., 1992; Molnar *et al*., 1993; Zhisheng *et al*., 2001). Most members of the subgenus *Paa* are distributed below 2500 m a.s.l.. Therefore, our result suggests that species formation may be associated with active orogeny within Tibet. All three species of the subgenus *Nanorana* dwell at altitudes from nearly 3000 m up to 4700 m a.s.l.. Members of this subgenus seem to have adapted progressively as the entire Tibetan plateau experienced significant increases in altitude to at least the 3000 m level (Che *et al*., 2010). Our results are compatible with the notion that although the species is today a high altitude native, it originated from the plain species, and it still has the ability to adapt to the lowland environment.

Plasticity might allow a population to survive long enough in a new environment for existing genetic variation, in combination with mutation and/or recombination, to respond to local selection conditions

**Table 4**  The results of independent samples test for each gene.

| Gene | Groups | Levene's test for equality of variances | | *t*-test for equality of means | | |
|---|---|---|---|---|---|---|
| | | *F* | *Sig.* | *t* | *df* | *Sig.*(2-tailed) |
| *HIF-1A* | Equal variances assumed | 13.21 | 0.022 | 8.279 | 4 | 0.001 |
| | Equal variances not assumed | | | 8.279 | 2.011 | 0.014 |
| *VEGF* | Equal variances assumed | 13.11 | 0.022 | 3.6 | 4 | 0.023 |
| | Equal variances not assumed | | | 3.6 | 2.029 | 0.048 |
| *EPAS1* | Equal variances assumed | 3.182 | 0.149 | 4.094 | 4 | 0.015 |
| | Equal variances not assumed | | | 4.094 | 2.061 | 0.052 |
| *EPO* | Equal variances assumed | 0.328 | 0.598 | −0.48 | 4 | 0.656 |
| | Equal variances not assumed | | | −0.48 | 3.785 | 0.658 |

(Johansson *et al.*, 2013). In our study, we observed phenotypic plasticity mediated by gene expression plasticity. To test whether genetic accommodation has occurred, we need to study genetic variation at the specific genes in the future. In addition, with today's technology, creating whole transcriptomes becomes affordable and might allow for a fresh look without being biased towards knowledge about metabolic pathways in other systems. Therefore, transcriptomic analysis by means of RNA sequencing may in future help us to identify currently unknown pathways that are specific to the Tibetan frog.

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