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ORIGINAL ARTICLE

Expression of HIF-1α and Its Target Genes in the *Nanorana parkeri* Heart: Implications for High Altitude Adaptation

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Abstract Hypoxia-inducible factor 1 alpha (HIF-1α) and its target genes vascular endothelial growth factor (*VEGF*) and transferrins (*TF*) play an important role in native endothermic animals' adaptation to the high altitude environments. For ectothermic animals – especially frogs – it remains undetermined whether HIF-1α and its target genes (*VEGF* and *TF*) play an important role in high altitude adaptation, too. In this study, we compared the gene sequences and expression of HIF-1α and its target genes (*VEGF* and *TF*) between three *Nanorana parkeri* populations from different altitudes (3008 m a.s.l., 3440 m a.s.l. and 4312 m a.s.l.). We observed that the cDNA sequences of *HIF-1A* exhibited high sequence similarity (99.38%) among the three altitudinally separated populations; but with increasing altitude, the expression of *HIF-1A* and its target genes (*VEGF* and *TF*) increased significantly. These results indicate that HIF-1α plays an important role in *N. parkeri* adaptation to the high altitude, similar to its role in endothermic animals.

Keywords Hypoxia, cold-temperature, ectothermic animals, *Nanorana parkeri*, high altitude, vascular endothelial growth factor, transferrins, anura, amphibia

1. Introduction

A high mountain range's plateau environment is hostile to life due to the low atmospheric oxygen pressure (up to about 40% lower than at sea level on the Tibetan plateau, for example), cold climate and strong ultraviolet radiation. Hypoxic conditions may compromise cell and organ metabolism; especially for the heart, because the heart is an obligate aerobic organ. Under hypoxic conditions, the heart muscle not only cannot produce enough energy to maintain essential cellular processes, but also may be subjected to cardiac dysfunction, ultimately leading to death (Giordano, 2005). Organisms with long-term adaptations to high altitude environments have evolved a set of specific physiological traits to survive in this harsh environment. The study of the evolutionary basis of adaptive mechanisms to alleviate hypoxia not only has important biological, but also clinical implications. This offers the opportunity to contribute to fundamental human medical research by means of evolutionary studies (Rose, 2001).

Endothermic animals native to high altitude areas, such as the domestic yak (*Bos grunniens*), plateau pika (*Ochotona curzoniae*) and the human Tibetan population have developed traits to survive in highly hypoxic environments. Examples for such adaptations are larger

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lung capacity, lower pulmonary arterial pressure, and higher haemoglobin concentration (Cruz *et al.*, 1980; Moore *et al.*, 2000; Li *et al.*, 2001; Wu and Kayser, 2006). Compared with endothermic animals, ectothermic animals-especially frogs-carry many special characteristics, such as the incomplete development of the respiratory and circulatory system, abundant skin secretion, and also pronounced hypoxia tolerance (Knickerbocker and Lutz, 2001; Stewart *et al.*, 2004). Previous studies showed that they have evolved a highly efficient and well-regulated metabolism to counter the impacts of extreme environmental conditions in the field. For example, *Telmatobius coleus*, one of the plateau anurans, harbours an increased skin surface area where the cutaneous capillaries penetrate to the outer layer of the skin, and has elevated haemoglobin concentration and haematocrit in comparison with sea-level anurans (Hutchison *et al.*, 1976).

The molecular mechanisms underlying these phenotypic traits are modulated by several specific genes. For example, vascular endothelial growth factor (*VEGF*) plays an important role in adaptation to high altitude hypoxia environments for plateau pika (Li *et al.*, 2013) and the Peruvian human population in the Andes (Espinoza *et al.*, 2014). Transferrins (encoded by *TF*) play an important role in iron transportation during erythropoiesis in Ethiopians (Beall *et al.*, 2002). Expression levels of egl nine homolog 1 (*EGLN1*) and peroxisome proliferator-activated receptor alpha (*PPARA*) were significantly associated with the decreased haemoglobin phenotype in Tibetan human populations (Simonson *et al.*, 2010). The ADAM metallopeptidase domain 17 (*ADAM17*), arginase 2 (*ARG2*) and matrix metalloproteinase-3(*MMP3*) genes were detected to be under positive selection in Yak (Qiu *et al.*, 2012), and chemokine (C-C motif) ligand 2 (*CCL2*) and pyruvate kinase isozymes R/L (*PKLR*) in Tibetan antelope (*Pantholops hodgsonii*; Ge *et al.*, 2013).

All genes mentioned above are parts of the hypoxia-inducible factor (HIF) pathway. HIFs are crucially involved in maintaining oxygen homeostasis. They 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). HIF-1α plays a critical role in transcriptional regulation of the amount and timing of targeted gene production during hypoxia, which mediates many genes involved in erythropoiesis, angiogenesis, autophagy, and energy metabolism (William and Peter, 2008). For example, HIF-1α regulates *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). *TF* encodes transferrins, which are other proteins modulated by HIF-1α. They mediate cellular iron uptake and deliver iron to cells requiring it (Tacchini *et al.*, 1999). Iron is essential for oxygen delivery, as it is incorporated in the newly synthesized haemoglobin throughout erythropoiesis. Therefore, HIF-1α is a key transcription factor that regulates a variety of cellular and systemic adaptations to hypoxia; *VEGF* and *TF* are pivotal target genes of HIF-1α in angiogenesis and erythropoiesis under hypoxia. Although physiological responses to hypoxia have been extensively studied in plateau frogs (e.g. Weber *et al.*, 2002), whether HIF-1α plays an important role in ranid adaptation to high elevation environments, like it does in endothermic animals, is poorly understood.

The Qinghai-Tibetan plateau (at greater than 4000 m a.s.l.) is the highest plateau in the world, which provides the best opportunity for us to study the adaptation of ectothermic animals to high altitude hypoxic environments in their natural habitat. *Nanorana parkeri* is an anuran endemic to the southern Tibetan plateau and distributes across a narrow latitudinal (28 to 31°N) but extensive altitudinal range (2850 to 5100 m a.s.l.). Therefore, *N. parkeri* represents the highest altitude ranid in the world (Hu, 1987). Across the species' altitudinal range, environmental conditions vary large, for example, annual mean temperature ranged from 3.0°C to 8.6°C; air oxygen content ranged from 88 to 114 mg/cm^3 (Zhang *et al.*, 2012). Although *N. parkeri* has been a model to study morphology, life history and biological chemistry in high altitude environments (Ma *et al.*, 2009; Ma and Lu, 2009, 2010; Lu *et al.*, 2010; Zhang *et al.*, 2012), the role of HIF-1α in their adaptation to high altitude remains undetermined. In this study, we compared the expression of HIF-1α and its target genes in *N. parkeri* in heart tissue (*VEGF* and *TF*) between populations of three different altitudes (low: 3008 m a.s.l., medium: 3440 m a.s.l., high: 4312 m a.s.l.) .

2. Materials and Methods

2.1 Sample preparation Healthy adult *Nanorana parkeri* were captured at various altitudes (3008 m a.s.l., 3440 m a.s.l. and 4312 m a.s.l.) in the Sejila Mountains, in Nyingtri county, Tibet in June 2014 (Table 1). Five individuals for each altitude were used for HIF-1α quantification. Animals were killed by double-pithing technique adopted from Costanzo *et al.* (1991) immediately upon capture to harvest heart tissue. Half of the tissue preserved in RNA holder (TransGen Biotech Co., Ltd., Beijing, China, stored at room temperature), was brought to our laboratory in Beijing and used for RNA extraction. The remaining tissue was frozen at –80°C and transferred to our laboratory for protein extraction. 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) for further analyses.

We designed *HIF-1A*, *VEGF* and *TF* primers 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 temporaria*), rainbow trout (*Oncorhynchus mykiss*), African clawed frog (*Xenopus laevis*) and tropicalis frog (*Xenopus tropicalis*) in GenBank (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. The total of 0.6 μg RNA isolated from *N. parkeri* heart for each altitude from each of five individuals were pooled into a total aliquot of three 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* and *TF* mRNA. The amplification was performed for 40 cycles at the 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 *β*-Actin generated from the same sample (Livak and Schmittgen 2001).

2.4 Sequence alignment The PCR products of *HIF-1A* of the three altitude groups were sequenced with an automated sequencer by the BGI Tech Solutions Corporation (Shenzhen, China). For each altitude, the PCR products from the cDNA of the pool of five individuals were sequenced together. Multiple sequence alignment was carried out using DNAMAN software package (Lynnon Biosoft).

Table 1 Samples information.

Location	Coordinates	Altitudes
Bayi, Nyingtri, Tibet	29.40° N, 94.19° E	3008 m
Lulang, Nyingtri, Tibet	29.42° N, 94.43° E	3440 m
Mainling, Nyingtri, Tibet	29.31° N, 94.37° E	4312 m

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
VEGF-F	TATCAAAGTCGCAAACC
VEGF-R	TATCCCACTGCCAACC
TF-F	TGATGACTTGGCAGAT
TF-R	CCATCCCATTTGAATA

2.5 Western blot Hearts of three samples (together 100 mg) from each altitude were homogenized in 1 ml lysis buffer (1 mM PMSF, 3 mM EDTA, 40 mM Tris (PH 7.5), 5 mM DTT). The tissue was crushed on ice, and centrifuged at 10 000 rpm (Sigma 1-15K, Germany) for 15 min at 4°C. Then the upper layer was transferred into a new 1.5 ml Eppendorf PE tube. Protein concentration was measured directly with a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., DE). An aliquot containing 30 μg of protein was diluted in loading buffer (loading buffer:sample = 5:1, v/v, heated to 97°C for 15 min) and was separated by 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) electrophoresis until the blue dye front was at the end of the gel but not diffused off the gel. Then, the protein was transferred onto a 0.45-μm-pore nitrocellulose filter membrane (NC, Immuno-Blot, BioRad, USA) at 9V for 1 h at 4°C. The membranes were blocked at room temperature for 1 h with 3 % fat-free milk in TBS (2M Tris, NaCl, PH 7.5). The membrane was then incubated in 1:500 diluted HIF-1α antibody (Abcam, Cambridge, UK) at 4°C overnight. After washing twice with TBS-T (1 L TBS + 200 μl Tween), and twice with TBS-every washing lasted for 10 min - the membrane was incubated with HIF-1α-ChIP grade antibody (diluted 1:10 000; AB2185, Abcam, Cambridge, MA) for 3h at room temperature. After additional washing, twice with TBS-T and twice with TBS, proteins were visualized by exposing the blot to an X-ray film, and photographed with an ImageQuant

LAS4000 (GE Healthcare UK Ltd, Little Chalfont, UK). The net intensities of individual bands were measured using Quantity One (version 4.6.2, Bio-Rad company, USA). Each altitude group was measured three times.

2.6 Statistical analysis of data Results were presented as mean ± S.E. per altitude group. Group means were compared by one-way analysis of variance, with a post hoc Scheffe's test. A value of $P < 0.05$ was considered statistically significant (SPSS ver. 17.0).

3. Results

3.1 The sequence alignment of *HIF-1A* Chromatograms of the pooled sequences indicated no mixed signals from nucleotide variation that might have been present in the pooled individuals. We therefore infer no signs of intra-altitude genetic variation. The length of the *N. parkeri HIF-1A* cDNA was 2358 bp. The identity of *HIF-1A* cDNA sequences between altitudes was 99.38% across the three altitudes (3008 m a.s.l., 3440 m a.s.l. and 4312 m a.s.l.; Figure 1), and there were a total number of 28 variable sites. Among them, 23 were substitutions, and five of them were indels (insertions or deletions). Eight of these substitution sites lead to amino acid differences (Table 3).

Table 3 Eight substitution sites cause amino acid differences.

Amino acid change	Altitude		
	Low	Medium	High
Y205C	Y	C	C
N811C	N	N	C
S814M	S	S	M
W817S	W	W	S
E820I	E	E	I
G823I	G	G	I
Q826T	Q	Q	T
H882M	H	M	M

3.2 The expression of HIF-1α protein The protein concentration of HIF-1α increased significantly with increasing altitude, as measured by the net intensities ± SE of individuals bands: 8.20 ± 0.8418 (low altitude), 24.81 ± 1.6079 (medium altitude), and 68.63 ± 1.0281 (high altitude) (Table 4 Line A, Figure 2A).

3.3 The expression of *HIF-1A*, *VEGF* and *TF* mRNA The expression of *HIF-1A*, *VEGF* or *TF* mRNA increased with altitude, too (Figure 2B, C, D). For *HIF-1A* and *VEGF*, the largest source of variance was between groups; for example, the expression of high altitude was significantly higher than the medium altitude and low altitude. For *TF*, the largest source of variance derived from within groups, so no significant differences among the three altitudes was observed (Table 4 Lines B, C, D).

4. Discussion

Although sequence similarity of *HIF-1A* among samples collected from the three altitudes was high, some substitutions have led to amino acid changes (Table 3). Furthermore, there seem to be more genetic differences between the high altitude group and the two other groups. For example, six of the eight amino acid changes were between high altitude and the two other altitude groups; therefore, the high altitude environment seems to have resulted in the largest change in the genetic background. Variation in the amino acid sequence may induce important functional changes of HIF-1α, and could therefore be responsible for differences between altitude groups. A functional analysis of the changed amino acid residues in further proteomic experiments might shed light on the important questions of the function of this protein.

Simultaneously, differential gene expression patterns among different altitude groups were observed. The HIF-1α expression of *N. parkeri* is increasing with increased habitat altitude. The same pattern of expression is also observed in plateau pika (*Ochotona curzoniae*; Li *et al*., 2009). These results indicate that *HIF-1A* is a hypoxia-inducible gene in *N. parkeri*, just like in endothermic animals. In lower vertebrates, the role of HIF-1α in hypoxia tolerance was first reported for rainbow trout (Soitamo *et al*., 2001). Furthermore, the role of HIF-1α in hypoxia tolerance has also been proven indirectly by a set of target genes of HIF-1α in euryoxic fish (*Gillichthys mirabilis*; Gracey *et al*., 2001). Rissanen *et al*. (2006) found that except for hypoxia (Cao *et al*. 2008), cold temperature also induces the expression of HIF-1α in crucian carp (Rissanen *et al*., 2006). In our study, low temperature and high altitude habitats covary, and temperature could thus play an additional role in altitude-related HIF-1α regulation. The possible interaction of altitude and temperature will need to be addressed in future experiments. However, whatever up-regulated the *HIF-1A* expression, hypoxia or the cold temperature, HIF-1α plays an important role in the local adaptation of *N. parkeri* to its high-altitude environment.

Our findings indicate that *VEGF* mRNA levels are increased in the *N. parkeri* that inhabit higher altitudes. The trend is similar to the changes in HIF-1α mRNA expression with altitude. It is well known that hypoxia-

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CLUSTAL multiple sequence alignment

HIF-1AH.txt      CGGCTGGCATGGAGGGAGCAGTAGTGACAGACAAGAAAAGGATCAGTTCGGAGCGGCGGA
HIF-1AM.txt      CGGCTGGCATGGAGGGAGCAGTAGTGACAGACAAGAAAAGGATCAGTTCGGAGCGGCGGA
HIF-1AL.txt      CGGCTGGCATGGAGGGAGCAGTAGTGACAGACAAGAAAAGGATCAGTTCGGAGCGGCGGA
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HIF-1AH.txt      AGGAGAAATCTCGGGATGCTGCAAGGTGTCGGAGGAGTAAAGAATCGGAGGTCTTTTATG
HIF-1AM.txt      AGGAGAAATCTCGGGATGCTGCAAGGTGTCGGAGGAGTAAAGAATCGGAGGTCTTTTATG
HIF-1AL.txt      AGGAGAAATCTCGGGATGCTGCAAGGTGTCGGAGGAGTAAAGAATCGGAGGTCTTTTATG
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HIF-1AH.txt      AACTGTCCCACCAGCTGCCGCTGCCTCACAATGTCAGCTCTCATCTTGATAAAGCCTCCA
HIF-1AM.txt      AACTGTCCCACCAGCTGCCGCTGCCTCACAATGTCAGCTCTCATCTTGATAAAGCCTCCA
HIF-1AL.txt      AACTGTCCCACCAGCTGCCGCTGCCTCACAATGTCAGCTCTCATCTTGATAAAGCCTCCA
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HIF-1AH.txt      TAATGAGGCTCGCCATCAGCTACCTGCGTCTAAGAAGGCTCCTCGATGCAGGTGAAGCAG
HIF-1AM.txt      TAATGAGGCTCGCCATCAGCTACCTGCGTCTAAGAAGGCTCCTCGATGCAGGTGAAGCAG
HIF-1AL.txt      TAATGAGGCTCGCCATCAGCTACCTACGTCTAAGAAGGCTCCTCGATGCAGGTGAAGCAG
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HIF-1AH.txt      CTGGAGAGTCTGACATGGAAAAACAGCTGAATTGTTTTTATCTAAAGGCCTTAGAGGGAT
HIF-1AM.txt      CTGGAGAGTCTGACATGGAAAAACAGCTGAATTGTTTTTATCTAAAGGCCTTAGAGGGAT
HIF-1AL.txt      CTGGAGAGTCTGACATGGAAAAACAGCTGAATTGTTTTTATCTAAAGGCCTTAGAGGGAT
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HIF-1AH.txt      TTGTTTTGGTCCTGACTGAGGAAGGGGATATGATCTACCTGTCTGAAAATGTCAACAAGT
HIF-1AM.txt      TTGTTTTGGTCCTGACTGAGGAAGGGGATATGATCTACCTGTCTGAAAATGTCAACAAGT
HIF-1AL.txt      TTGTTTTGGTCCTGACTGAGGAAGGGGATATGATCTACCTGTCTGAAAATGTCAACAAGT
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HIF-1AH.txt      GCATGGGACTCACACAGTTTGAGCTGACTGGGCACAGTGTGTTCGACTTCACCCACCCCT
HIF-1AM.txt      GCATGGGACTCACACAGTTTGAGCTGACTGGGCACAGTGTGTTCGACTTCACCCACCCCT
HIF-1AL.txt      GCATGGGACTCACACAGTTTGAGCTGACTGGGCACAGTGTGTTCGACTTCACCCACCCCT
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HIF-1AH.txt      GTGATCATGAGGAGCTGAGGGAGACGCTGACATTTAGAAATGGACCAGCAAAGAAGGGTA
HIF-1AM.txt      GTGATCATGAGGAGCTGAGGGAGACGCTGACATTTAGAAATGGACCAGCAAAGAAGGGTA
HIF-1AL.txt      GTGATCATGAGGAGCTGAGGGAGACGCTGACATTTAGAAATGGACCAGCAAAGAAGGGTA
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HIF-1AH.txt      AAGAGCAAATCACAGAGCGCAGCTTCTTCCTGCGTATGAAGTGCACCCTCACAAGCCGGG
HIF-1AM.txt      AAGAGCAAATCACAGAGCGCAGCTTCTTCCTGCGTATGAAGTGCACCCTCACAAGCCGGG
HIF-1AL.txt      AAGAGCAAATCACAGAGCGCAGCTTCTTCCTGCGTATGAAGTGCACCCTCACAAGCCGGG
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HIF-1AH.txt      GGAGAACCGTAAATATCAAGTCTGCCACGTGGAAGGTTCTTCACTGCACAGGGCACATGC
HIF-1AM.txt      GGAGAACCGTAAATATCAAGTCTGCCACGTGGAAGGTTCTTCACTGCACAGGGCACATGC
HIF-1AL.txt      GGAGAACCGTAAATATCAAGTCTGCCACGTGGAAGGTTCTTCACTGCACAGGGCACATGC
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HIF-1AH.txt      GTGTGTATGACAACTGCAACAACCAGAGCCATTGCGGATATAAGAAGCCACCCATGACCT
HIF-1AM.txt      GTGTGTATGACAACTGCAACAACCAGAGCCATTGCGGATATAAGAAGCCACCCATGACCT
HIF-1AL.txt      GTGTGTATGACAACTGCAACAACCAGAGCCATTGCGGATATAAGAAGCCACCCATGACCT
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HIF-1AH.txt      GCATGGTGCTGATCTGCGAACCTATCCCTCACCCATCAAATATTGAATTTCCATTGGACA
HIF-1AM.txt      GCATGGTGCTGATCTGCGAACCTATCCCTCACCCATCAAATATTGAATTTCCATTGGACA
HIF-1AL.txt      GCATGGTGCTGATCTGCGAACCTATCCCTCACCCATCAAATATTGAATTTCCATTGGACA
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HIF-1AH.txt      GTAAAACCTTCCTGAGCCGCCACAGCCTTGACATGAAGTTCTCTTATTGTGACGAAAGAG
HIF-1AM.txt      GTAAAACCTTCCTGAGCCGCCACAGCCTTGACATGAAGTTCTCTTATTGTGACGAAAGAG
HIF-1AL.txt      GTAAAACCTTCCTGAGCCGCCACAGCCTTGACATGAAGTTCTCTTATTGTGACGAAAGAG
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HIF-1AH.txt      TTACAGAGCTGGCAGGATATGAGCCAGATGTGTATGAGTATTATCACGCCGTGTATGAGT
HIF-1AM.txt      TTACAGAGCTGGCAGGATATGAGCCAGATGAACTCCTGGGAAGGT---CAGTGTATGAGT
HIF-1AL.txt      TTACAGAGCTGGCAGGATATGAGCCAGATGAACTCCTGGGAAGGT---CAGTGTATGAGT
                 ******************************       *          * **********

HIF-1AH.txt      ATTATCACGCCCTGGACTCTGACCACCTGACCAAAGCACACC-ATGACATGTTCACCAAA
HIF-1AM.txt      ATTATCACGCCCTGGACTCTGACCACCTGACCAAAGCACACC-ATGACATGTTCACCAAA
HIF-1AL.txt      ATTATCACGCCCTGGACTCTGACCACCTGACCAAAGCACACCCATGACATGTTCACCAAA
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HIF-1AH.txt      GGGCAGGTGACAACAGGACAGTACAGGCTGCTGGCCAAGAAAGGTGGCTATGTCTGGGTG
HIF-1AM.txt      GGGCAGGTGACAACAGGACAGTACAGGCTGCTGGCCAAGAAAGGTGGCTATGTCTGGGTG
HIF-1AL.txt      GGGCAGGTGACAACAGGACAGTACAGGCTGCTGGCCAAGAAAGGTGGCTATGTCTGGGTG
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HIF-1AH.txt      GAGACACAGGCCACGGTTATATACAATACAAAGAACTCCCAGCCCCAGTGTATTGTGTGC
HIF-1AM.txt      GAGACACAGGCCACGGTTATATACAATACAAAGAACTCCCAGCCCCAGTGTATTGTGTGC
HIF-1AL.txt      GAGACACAGGCCACGGTTATATACAATACAAAGAACTCCCAGCCCCAGTGTATTGTGTGC
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HIF-1AH.txt      GTGAACTATGTCCTCAGTGGCATTGTGGAGAACGAGCTGGTCTTATCCCTTGGCCAGACA
HIF-1AM.txt      GTGAACTATGTCCTCAGTGGCATTGTGGAGAACGAGCTGGTCTTATCCCTTGGCCAGACA
HIF-1AL.txt      GTGAACTATGTCCTCAGTGGCATTGTGGAGAACGAGCTGGTCTTATCCCTTGGCCAGACA
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HIF-1AH.txt      GAGTCCACAGAATCTGCTGAAATAAAGATGCCGCAGATCTTCACTAAACTGGATGTGGAG
HIF-1AM.txt      GAGTCCACAGAATCTGCTGAAATAAAGATGCCGCAGATCTTCACTAAACTGGATGTGGAG
HIF-1AL.txt      GAGTCCACAGAATCTGCTGAAATAAAGATGCCGCAGATCTTCACTAAACTGGATGTGGAG
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HIF-1AH.txt      GAGGACACGGAAAGCCTGTTTGACAAACTGAAAAAGGAGCCGGAGTCACTGACTGTTCTT
HIF-1AM.txt      GAGGACACGGAAAGCCTGTTTGACAAACTGAAAAAGGAGCCGGAGTCACTGACTGTTCTT
HIF-1AL.txt      GAGGACACGGAAAGCCTGTTTGACAAACTGAAAAAGGAGCCGGAGTCACTGACTGTTCTT
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HIF-1AH.txt      GCACCTGATGCTGGAGATGAGATCATAGCCTTGGACTTCAGTTCCAGTGATTCAGAGCCG
HIF-1AM.txt      GCACCTGATGCTGGAGATGAGATCATAGCCTTGGACTTCAGTTCCAGTGATTCAGAGCCG
HIF-1AL.txt      GCACCTGATGCTGGAGATGAGATCATAGCCTTGGACTTCAGTTCCAGTGATTCAGAGCCG
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HIF-1AH.txt      CAGTTTGACGATGTCCCACTGTATAATGACGTCATGATGCACCCAACCAGTAAGCCTCCA
HIF-1AM.txt      CAGTTTGACGATGTCCCACTGTATAATGACGTCATGATGCACCCAACCAGTAAGCCTCCA
HIF-1AL.txt      CAGTTTGACGATGTCCCACTGTATAATGACGTCATGATGCACCCAACCAGTAAGCCTCCA
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HIF-1AH.txt      GAAAACCTGGTGTCTCCGCTTCCAGCGTTAGAGAAGCCGAAGCCTATGCGTAGCAATGCC
HIF-1AM.txt      GAAAACCTGGTGTCTCCGCTTCCAGCGTTAGAGAAGCCGAAGCCTATGCGTAGCAATGCC
HIF-1AL.txt      GAAAACCTGGTGTCTCCGCTTCCAGCGTTAGAGAAGCCGAAGCCTATGCGTAGCAATGCC
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HIF-1AH.txt      GACCCTGCTCTGAACAGGGAGGTGGTGATCAAGATGGAGACTAGCCCACAACAGCTGGCA
HIF-1AM.txt      GACCCTGCTCTGAACAGGGAGGTGGTGATCAAGATGGAGACTAGCCCACAACAGCTGGCA
HIF-1AL.txt      GACCCTGCTCTGAACAGGGAGGTGGTGATCAAGATGGAGACTAGCCCACAACAGCTGGCA
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HIF-1AH.txt      CTGGCTTTTACCATTCCACAGCTCTCCAAGCCATCTAGTCCAACAGAAATCAGCAGCAGC
HIF-1AM.txt      CTGGCTTTTACCATTCCACAGCTCTCCAAGCCATCTAGTCCAACAGAAATCAGCAGCAGC
HIF-1AL.txt      CTGGCTTTTACCATTCCACAGCTCTCCAAGCCATCTAGTCCAACAGAAATCAGCAGCAGC
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HIF-1AH.txt      CAGAGCTCAACGGAGCCCTGGCACTCCAGCAGAATATTGTTTCGATGTGGATAGTGAGAT
HIF-1AM.txt      CAGAGCTCAACGGAGCC-TGGCACTCCAGCAGAATATTGTTTCGATGTGGATAGTGAGAT
HIF-1AL.txt      CAGAGCTCAACGGAGCC-TGGCACTCCAGCAGAATATTGTTTCGATGTGGATAGTGAGAT
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HIF-1AH.txt      CTCCCGGGAATTTAAGATGGACTTGGTTGAGAAACTTTTTGCCATTGACACAGAAGCAAA
HIF-1AM.txt      CTCCCGGGAATTTAAGATGGACTTGGTTGAGAAACTTTTTGCCATTGACACAGAAGCAAA
HIF-1AL.txt      CTCCCGGGAATTTAAGATGGACTTGGTTGAGAAACTTTTTGCCATTGACACAGAAGCAAA
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HIF-1AH.txt      GACTCCATTCTGTACACAGGAAACAGACTTGGACCTGGAAATGTTGGCTCCATATATCCC
HIF-1AM.txt      GACTCCATTCTGTACACAGGAAACAGACTTGGACCTGGAAATGTTGGCTCCATATATCCC
HIF-1AL.txt      GACTCCATTCTGTACACAGGAAACAGACTTGGACCTGGAAATGTTGGCTCCATATATCCC
                 ************************************************************

HIF-1AH.txt      AATGGACGATGACTTCCAGCTAAGAAGCTTTGACCAGCTTTCCTCCATGGAGACTGATTC
HIF-1AM.txt      AATGGACGATGACTTCCAGCTAAGAAGCTTTGACCAGCTTTCCTCCATGGAGACTGATTC
HIF-1AL.txt      AATGGACGATGACTTCCAGCTAAGAAGCTTTGACCAGCTTTCCTCCATGGAGACTGATTC
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HIF-1AH.txt      CACTCATCCGCAGTCACTAGAAAGCATGACCAATCGTGCCAGGCCATCTACTGCTCCACC
HIF-1AM.txt      CACTCATCCGCAGTCACTAGAAAGCATGACCAATCTGTTCCAGCCATCTACTGCTCCACC
HIF-1AL.txt      CACTCATCCGCAGTCACTAGAAAGCATGACCAATCTGTTCCAGCCATCTACTGCTCCACC
                 ***********************************    *  ******************

HIF-1AH.txt      CATTACCGATCTGAAAGCAATCGCCACTGAAGCCATGAGCGATTTGGAAACCATCATTGT
HIF-1AM.txt      CATTACCGATCTGAAAGCAATCGCCACTGAAGCCATGAGCGATTTGGAAACCATCATTGT
HIF-1AL.txt      CATTACCGATCTGAAAGCAATCGCCACTGAAGCCATGAGCGATTTGGAAACCATCATTGT
                 ************************************************************

HIF-1AH.txt      GCACTCGCCTACACCCACCCAATAAAAAGGTGCTAAGTGCCCCCGCTTCCCCATATAATG
HIF-1AM.txt      GCACTCGCCTACACC-ACCCAATAAAAAGGAGCTAAGTGCCCCCGCTTCCCCATATAATG
HIF-1AL.txt      GCACTCGCCTACACCCACCCAATAAAAAGGTGCTAAGTGCCCCCGCTTCCCCATATAATG
                 *************** ************** *****************************

HIF-1AH.txt      CAAACCGAAGTCGGACAAGTTCTCCCGTGAGGACAGAGAAAGCAGCCGAAAAGGACAGAT
HIF-1AM.txt      CAAACCGAAGTCGGACAAGTTCTCCCGTGAGGACAGAGAAAGCAGCCGAAAAGGACAGAT
HIF-1AL.txt      CAAACCGAAGTCGGACAAGTTCTCCCGTGAGGACAGAGAAAGCAGCCGAAAAGGACAGAT
                 ************************************************************

HIF-1AH.txt      CCCGCCCTGGAACTCCCAATTTAACGAGTTCGCTAAATAAAAGACCTGCACCCATGGATG
HIF-1AM.txt      CCCGCCCTGGAACTCCCAATTTAACGAGTTCGCTAAATAAAAGACCTGCACCCATGGATG
HIF-1AL.txt      CCCGCCCTGGAACTCCCAATTTAACGAGTTCGCTAAATAAAAGACCTGCACCCATGGATG
                 ************************************************************

HIF-1AH.txt      ACGAAATGAGCCCAAAGATGATCGCTTTACATAATGTCCAGAGAAAACGCAAAATTGAAA
HIF-1AM.txt      ACGAAATGAGCCCAAAGATGATCGCTTTACATAATGTCCAGAGAAAACGCAAAATTGAAA
HIF-1AL.txt      ACGAAATGAGCCCAAAGATGATCGCTTTACATAATGTCCAGAGAAAACGCAAAATTGAAA
                 ************************************************************

HIF-1AH.txt      ACGATGGGCCTTTGTTTCAAGCAGTGGGACTAGGAACGTTATTCCAAACGAATGTTAATC
HIF-1AM.txt      ACGATGGGCCTTTGTTTCAAGCAGTGGGACTAGGAACGTTATTCCAAACGAATGTTAATC
HIF-1AL.txt      ACGATGGGCCTTTGTTTCAAGCAGTGGGACTAGGAACGTTATTCCAAACGAATGTTAATC
                 ************************************************************

HIF-1AH.txt      CAGGGCCTAACTCTCCTCTGTTATGGAAATGTGTCAAAGTGTCAGACTCTGATAAGCCAA
HIF-1AM.txt      CAGGGCCTAACTCTCCTCTGTTATGGAAATGTGTCAAAGTGTCAGACTCTGATAAGCCAA
HIF-1AL.txt      CAGGGCCTAACTCTCCTCTGTTATGGAAATGTGTCAAAGTGTCAGACTCTGATAAGCCAA
                 ************************************************************

HIF-1AH.txt      CTGGCCCTGAACAACGAACAATCCTCTTATTGTCTACAGATATGGCCAGTCGATTGCTTG
HIF-1AM.txt      CTGGCCCTGAACAACGAACAATCCTCTTATTGTCTACAGATATGGCCAGTCGATTGCTTG
HIF-1AL.txt      CTGGCCCTGAACAACGAACAATCCTCTTATTGTCTACAGATATGGCCAGTCGATTGCTTG
                 ************************************************************

HIF-1AH.txt      GACAGTCGTTGGATGGCACCAGGCTTCCTCAGCTAACTAGCTACGACTGCGAAGTGAACG
HIF-1AM.txt      GACAGTCGTTGGATGGCACCAGGCTTCCTCAGCTAACTAGCTACGACTGCGAAGTGAACG
HIF-1AL.txt      GACAGTCGTTGGATGGCACCAGGCTTCCTCAGCTAACTAGCTACGACTGCGAAGTGAACG
                 ************************************************************

HIF-1AH.txt      CCCCGATACAAGGAAGCCG
HIF-1AM.txt      CCCCGATACAAGGAAGC--
HIF-1AL.txt      CCCCGATACAAGGAAGC--
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Figure 1 Multiple sequence alignment of *Nanorana parkeri* HIF-1α cDNA at three altitudes (high altitude: HIF-1AH, medium altitude: HIF-1AM and low altitude: HIF-1AL). Asterisks indicate identical sites and the gap indicated the variable sites among three altitude sequences.

Table 4 The ANOVA results of HIF-1α protein and *HIF-1A*, *VEGF* and *TF* mRNA expression.

		Sum of Squares	*df*	Mean Square	*F*	*Sig.*
	Between Groups	5847.688	2	2923.844	671.976	0
A. HIF-1α	Within Groups	26.107	6	4.351		
	Total	5873.795	8			
	Between Groups	17.822	2	8.911	29.398	0.001
B. *HIF-1A*	Within Groups	1.819	6	0.303		
	Total	19.64	8			
	Between Groups	1.368	2	0.684	13.743	0.006
C. *VEGF*	Within Groups	0.299	6	0.05		
	Total	1.667	8			
	Between Groups	0.082	2	0.041	0.134	0.877
D. *TF*	Within Groups	1.841	6	0.307		
	Total	1.924	8			

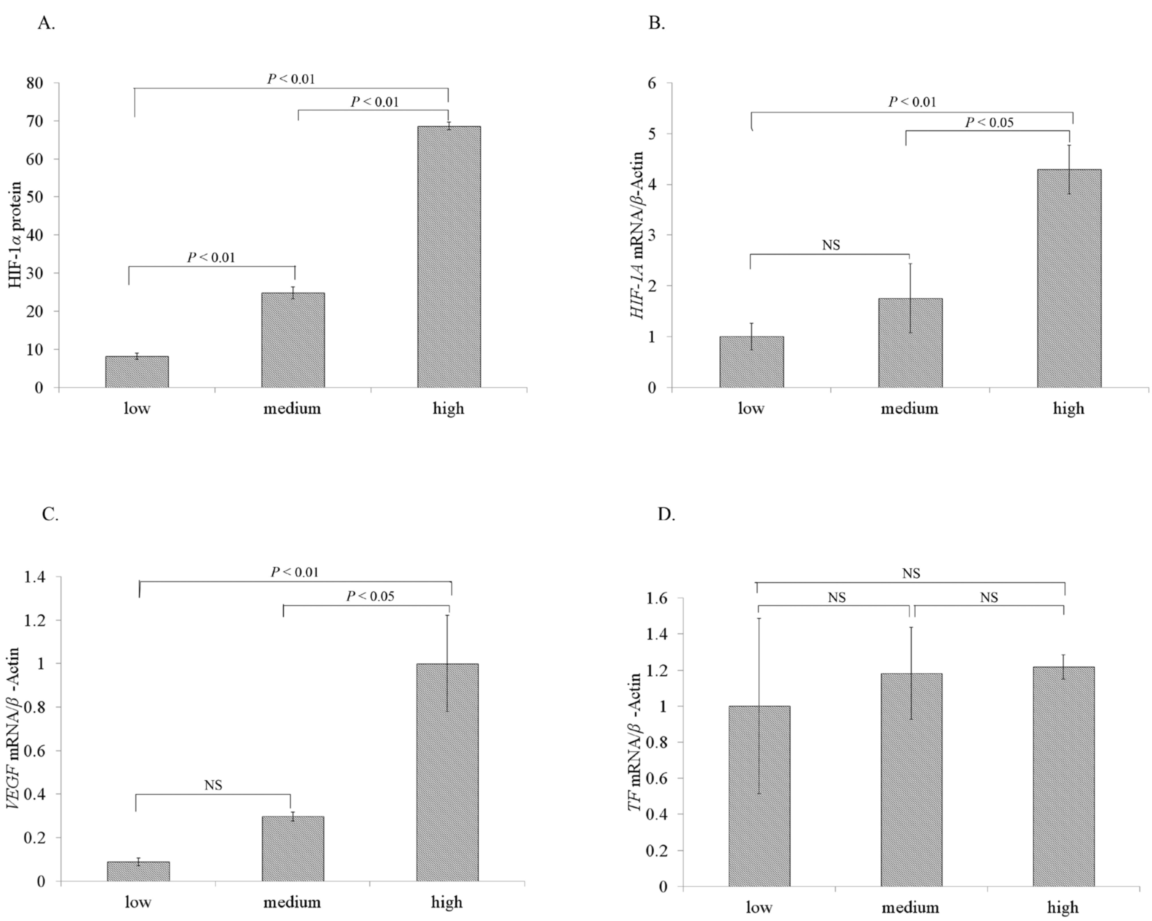

Figure 2 Expression of HIF-1α protein (A) and *HIF-1A* (B) , *VEGF* (C) and *TF* (D) mRNA of *Nanorana parkeri* at different altitudes (low, 3008 m; medium, 3440 m; high, 4312 m). For mRNA, expression levels were normalized to *β-actin* mRNA levels. Representative results from three independent experiments in triplicate on the same protein or mRNA of different individuals are presented as means ± standard error.

induced expression of *VEGF* is under the control of *HIF-1A* in other species (Damert *et al.*, 1997), therefore we assume that the higher expression level of *VEGF* mRNA may be supported by the higher expression of HIF-1α protein in *N. parkeri* inhabiting higher altitudes. In addition, low temperature is reported to be involved in angiogenesis through up-regulating *VEGF* expression by HIF in mouse adipose tissue (Xue *et al*., 2009). Thus, cold temperatures could also a play an important role in *VEGF* up-regulation, like for *HIF-1A*. Therefore, hypoxia and cold temperature, the two prime ecological factors of high-altitude habitat, may play an important role in the adaption of *N. parkeri* to high altitude environments through *HIF-1A* and *VEGF*.

Chytridiomycosis is a potentially lethal disease of amphibians caused by the amphibian chytrid fungus (*Batrachochytrium dendrobatidis*) that has been associated with population declines in several amphibian species throughout the world (Daszak *et al*., 1999; Carey, 2000; Green *et al*., 2002; Lips *et al*., 2006). Research suggests that *B. dendrobatidis* is more abundant in medium and high altitudes than low altitude because medium and high altitudes provide ideal temperatures for *B. dendrobatidis* (Daszak *et al*., 2003; Berger *et al*., 2004; Woodhams and Alford, 2005; Drew *et al*., 2006). The downstream gene of *HIF-1A*, *TF*, is up-regulated under hypoxic conditions in endothermic animals (e.g. Ethiopians, Beall *et al*., 2002; plateau pika, *Ochotona curzoniae,* Li *et al*., 2013). In our study, we indeed found a trend of increasing *TF* mRNA expression with increasing altitude in *N. parkeri*. *TF* is also associated with the innate immune system (Breitman *et al*., 1980; Evans *et al*., 1989; Stafford and Belosevic, 2003) as an acute phase protein in response to infection or stress conditions and limits the amount of iron, leading to the inhibition of bacterial growth (Sahoo *et al*., 2009). Based on the fact that orthologs of *TF* were identified in amphibians (Moskaitis *et al*., 1990; Morabito and Moczydlowski, 1994; Mohd-Padil H *et al*., 2012) and that the amphibian's skin can excrete antimicrobial peptides (Bevins and Zasloff, 1990), we hypothesize that high expression of *TF* mRNA could be related to defense mechanisms against pathogenic microorganisms in high altitude.

In conclusion, comparison of HIF-1α protein and mRNA expression across various altitudes indicates the important role of HIF-1α in adaptation to a high altitude environment. Our study made the first step for the understanding of ranids' adaptation to such high altitude environments. In future, creating whole transcriptomes (Wolf, 2013) will become affordable also for ecologically oriented working groups and might allow for a fresh look without being biased towards knowledge from other systems. Candidate genes for adaptive processes have been mined with genome-wide technology before in similar experimental or empirical set-ups (Bonin *et al*., 2006; Kane and Rieseberg, 2007) and true RNA sequencing may help us to identify so far unknown genes and pathways.

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