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ORIGINAL ARTICLE

Function of Lactate Dehydrogenase in Cardiac and Skeletal Muscle of *Phrynocephalus* Lizard in Relation to High-Altitude Adaptation

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Abstract Poikilothermic animals living in high-altitude environments can be greatly affected by the anaerobic metabolism and lactate recycling, which are catalyzed by an enzyme called lactate dehydrogenase (LDH). However, the function and possible regulatory mechanisms of their anaerobic glycolysis remained elusive. We compared the difference in LDH between a native high-altitude (4 353 m) lizard, *Phrynocephalus erythrurus,* and a closely related species, *Phrynocephalus przewalskii* that lives in intermediate altitude environment (1 400 m). The activity of LDH, the concentration of lactate, the distribution of isoenzyme, and the mRNA amounts of Ldh-A and Ldh-B were determined. In cardiac muscle, the lactate-forming activity of *P.erythrurus* in LDH was higher than of *P. przewalskii* LDH at all three temperatures tested (10 °C, 25 °C and 35 °C), while lactate-oxidation activity of LDH was significantly different between the two species only at 25 °C and 35 °C. In skeletal muscle, both lactate-forming and lactate-oxidation rates of *P. erythrurus* were lower than that of *P. przewalskii*. There was a higher proportion of H subunit and a significantly higher expression of Ldh-B, with a concomitant decrease of lactate concentration in *P. erythrurus*. These results indicate that *P. erythrurus* may have a strong potential for anaerobic metabolism, which is likely adapted to the hypoxic environment at high altitudes. Furthermore, *P. erythrurus* is capable of oxidizing more lactate than *P. przewalskii*. The Ldh-A cDNA of the two species consists of a 999 bp open reading frame (ORF), which encodes 332 amino acids, while Ldh-B cDNA consists of a 1 002 bp ORF encoding 333 amino acids. LDHA has the same amino acid sequence between the two species, but three amino acid substitutions (V12I, N21S and N318K) were observed in LDHB. Structure analysis of LDH indicated that the substitutions of residues Val12 and Asp21 in *P. erythrurus* could be responsible for the high-altitude adaptation. The LDH characteristics of LDH in *P.erythrurus* suggest unique adaptation strategies of anaerobic metabolism in hypoxia and cold environments at high altitudes for poikilothermic animals.

Keywords lactate dehydrogenase (LDH), high altitude, adaptation, anaerobic metabolism, *Phrynocephalus erythrurus*

1. Introduction

High-altitude environments present a formidable challenge to humans and other animals, because of the low levels of O_2 and cold temperatures (Storz *et al*., 2010). High-altitude hypobaric hypoxia is an unavoidable severe stress that is difficult to mitigate (e.g. by behavior), and yet numerous species have successfully acquired adaptive regulatory mechanisms to thrive in these environment by minimizing performance decrements (Bickler and Buck 2007, Tang *et al*., 2013). Documented adaptations include morphological traits, hematological characteristics, thermogenesis, and metabolism involving both genetic and phenotypic levels (Sheafor, 2003; Storz *et al*., 2010; Scott *et al*., 2011). Given that anaerobic metabolism plays an important role in energy supply

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and regulation, increasing attention is being paid to the metabolic adaptation under hypoxic conditions. Lactate is an oxidative substrate for energy metabolism (Schurr, 2006), and lactate dehydrogenase (LDH, E.C. 1.1.1.27), which catalyzes the reversible conversion between lactate and pyruvate, commonly is used to study the anaerobic metabolism of high-altitude animals. The regulation and modification of LDH have been investigated under different temperatures and hypoxia by using physiological, biochemical (enzyme activity, isozyme distribution), and genetic strategies (molecular structure, amino acid sequence substitutions) (Rossignol *et al*., 2003; Selvakumar and Geraldine, 2003; Fields and Houseman, 2004; Johns and Somero, 2004; Hoff *et al*., 2016). LDH is a tetrameric enzyme composed of H (heart) and M (muscle) subunits, which are encoded in reptiles by the genes Ldh-A and Ldh-B, respectively (Mannen *et al*., 1997; Liao *et al*., 2001). Furthermore, the LDH tetramer has five isoenzymes, each differentiated by the H/M subunit composition M_4 (A_4, LDH_5), M_3H_1 (A_3B_1, LDH_4), M_2H_2 (A_2B_2, LDH_3), M_1H_3 (A_1B_3, LDH_2) and H_4 (B_4, LDH_1) (Markert *et al*., 1975; Ji *et al*., 1986). The isoenzyme distribution in different tissues can be influenced by hormonal changes (Kaaja and Are, 1996), developmental patterns (Bushong, 1966), cold exposure (Kaaja and Are, 1982), and most importantly, hypoxia (Anderson and Bullard 1971; Binette *et al*., 1977; Kaaja and Are, 1982; Rossignol *et al*., 2003). Numerous *in vivo* studies have shown that distribution patterns, catalytic properties, substrate affinities and inhibition of the five LDH isoenzymes, and their expression patterns reflect different metabolic roles in different tissues (Cahn *et al*., 1962; Krieg *et al*., 1967; Jr and Hale, 1968; Mager *et al*., 1968; Ross *et al*., 2010).

LDH activity has been extensively studied for years. The activities vary greatly among organs and tissues, and respond differently under hypoxic conditions. During hypoxia or moderate intensity exercise, LDH in skeletal muscle catalyzes the conversion of pyruvate to lactate. The lactate produced likely exits from muscles through monocarboxylate transport protein (Stanley *et al*., 1988) and is subsequently used as the primary fuel source for the mitochondrial TCA cycle of heart (Schurr, 2006). Nevertheless, skeletal muscle is not only responsible for the lactate production but also for the removal of lactate from the circulation (Drury and Wick, 1956). It seems lactate is a crucial fuel for mitochondrial respiration in skeletal muscles (Schurr, 2006). This regulation, which may contribute to the energy supply of skeletal muscle, is a reasonable explanation of the often observed down-regulation of LDH activity with reduced lactate level in high-altitude species, which is referred as "the lactate paradox" (Yang *et al*., 2008; Zhang *et al*., 2008; Noakes, 2009). However, LDH activity was found to compensate for the decay of oxidative energetic metabolism in hypoxic conditions (Rossignol *et al*., 2003). Therefore, it is still unknown if the controversial theory of "The lactate paradox" can be applicable to high-altitude poikilothermic animals or not.

LDH, especially in enzyme catalysis, has recently been widely used as a model enzyme to study the structure–function relationships associated with biochemical adaptation to hypoxic environments, (Rees *et al*., 2001; Johns and Somero, 2004; Fields *et al*., 2008; Soñanez-Organis *et al*., 2012). Though the amino acid sequences of LDHs across speices vary widely, their protein structures are highly conserved. For example, studies of *Amblyrhynchus cristatus* and *Coryphaenoides armatus* showed that amino acid substitutions in specific regions make this enzyme more suitable at high pressure of deep sea and across a broad range of ambient temperatures (Brindley *et al*., 2008; Fields *et al*., 2008). But unfortunately, for those lizards living at high altitudes, the structure-function relationships of their LDHs in high altitude living lizards have been rarely studied.

In contrast to avian and mammalian species, poikilothermic vertebrates exhibit a wide range of biochemical and physiological adaptations to the tremendous changes of O_2 availability and other environmental factors. The Red-tailed Toad-headed Lizard *Phrynocephalus erythrurus* (Lacertilia: Agamidae), which lives at 4 500–5 300 m on the Qinghai–Tibet Plateau, is considered to be the species living at the highest altitude of the world (Zhao and Adler, 1993). There has been no report so far about the function and possible regulation mechanisms of LDH adaptation in heart and skeletal muscle of this species. It is possible that the LDHs of lizards living at different altitudes have similar functions in the path-ways of anaerobic metabolism. However, we are more interested in the effect of hypoxia and low temperature environment on anaerobic metabolism and LDH function of the lizards living at high altitude. We therefore hypothesized that reptilian species living at high altitudes may possess more specialized and enhanced anaerobic metabolism than reptiles living at relatively low altitudes. Furthermore, it is unclear whether lactate shuttle exists in heart and skeletal muscle in Phrynocephalus lizards, and whether the lactate paradox is seen in high altitude living lizards. To test these hypotheses, we have compared the difference of the LDH activity of forward

and reverse reactions, LDH isoenzyme distribution, the sequences of Ldh-A and Ldh-B and their mRNA levels between *P. erythrurus* and *P. przewalskii* (low altitude lizard, 1 400 m) heart and skeletal muscles. We also analyzed the amino acid sequences of LDHA and LDHB, and constructed the three-dimensional (3D) models of the monomeric and tetrameric structures.

2. Materials and Methods

The animal utility protocol for this study was approved by the Ethics Committee of Animal Experiments at Lanzhou University, and is in accordance with guidelines from the China Council on Animal Care. Every effort was made to minimize the numbers used and animal suffering during the experiments.

2.1. Animals and sample collection *Phrynocephalus erythrurus* individuals (mean body mass: 5.11 ± 0.35 g; mean snout–vent length: 5.15 ± 0.11 cm) were captured in late July 2017 from near the Tuotuo River (34° 13' N, 92° 13' E; 4 543 m a. s. l.), Qinghai province. Individuals of a closely related species, *P. przewalskii* (mean body mass: 6.69 ± 0.29 g; mean snout–vent length: 5.80 ± 0.09 cm), were collected in June from Jingtai (34° 13' N, 103° 05' E; 1 400 m a. s. l.), Gansu province. In order to eliminate the possible effect of altitude, tissues of *P. erythrurus* were collected in its habitat, while *P. przewalskii* tissues were excised when brought to the laboratory at Lanzhou University (36° 05' N, 103° 86' E; 1 400 m a. s. l.). Climatic data of the two sampling sites were shown in the Table 1. The data were recorded at meteorological stations situated at or near (< 2 km) the collection sites. Lizards were anaesthetized by diethyl ether. Samples of heart and skeletal muscle were immediately dissected, weighed, snap-froze in liquid nitrogen, and stored at –80 °C for later analysis.

2.2. LDH enzyme activity, lactate concentration, and isoenzyme distribution All frozen tissues were quickly weighed and homogenized in nine volumes (w/v) of ice-cold extraction buffer (100 mmol/L KH_2PO_4/K_2HPO_4, pH 7.0) using refrigerated ground glass tissue homogenizers. The homogenates were then centrifuged at 9 000 g for 10 min. Supernatants were collected and used for LDH activity, lactate concentration, and isozyme distribution assays.

LDH activity for the forward reaction was measured using the method of Seebacher *et al.* (Seebacher *et al*., 2009). The supernatants were diluted by a factor of ten in extraction buffer and the reaction was started by adding 10 μL of diluted supernatant to 1 mL assay medium (100 mmol/L KH_2PO_4/K_2HPO_4, pH 7.0, 0.16 mmol/L NADH, 0.4 mmol/L pyruvate) pre-set to the desired temperature. The reverse reaction was measured according to a published protocol (Xiong and Storey, 2012). Assays were started by the addition of 10 μL diluted supernatant to 1 mL assay medium (100 mmol/L KH_2PO_4/K_2HPO_4, pH 8.0, 3 mmol/L NAD+, 22.5 mmol/L L-lactate). Enzyme activity was measured by monitoring changes in NADH absorbance at 340 nm, which was measured at 5 s intervals for 2 min (Gleeson and Harrison, 1986) using a TU-1901 UV-Vis spectrophotometer (PGeneral, Beijing). Assay temperature was set at 10, 25 and 35 °C, representing the lowest temperature in the burrow, body temperature when the lizards begin their activity (leaving burrows), and optimal selected body temperature of *P. erythrurus*, respectively. The temperature was controlled with a circulating water bath equipment on the cuvette holder. Enzyme activity was expressed as units per gram of protein to minimize the difference in protein concentrations and homogenization efficiency. Thermal sensitivity of the enzyme was expressed as Q_{10}, calculated by: $Q_{10} = (k_2/k_1)^{10/(T2-T1)}$, where k = reaction rate at temperature 1 and temperature 2 and T = temperature. Lactate concentration in heart and skeletal muscle was measured using an enzymatic assay (Kit A019-2, Nanjing Jiancheng Bioengineering Institute) and expressed as mg/g protein.

LDH isoenzymes were separated and characterized by native polyacrylamide gel electrophoresis on a vertical gel electrophoresis system (Liuyi, Beijing, China). Supernatants of the samples were diluted with 6× gel-loading buffer (0.1% bromophenol blue, 15% glycerol and 20 mmol/L Tris-Cl, pH 8.0) then loaded into a stacking gel of 5% acrylamide in 0.5 mmol/L Tris-Cl (pH 6.8). Electrophoresis was conducted at 80 V in a stacking gel and set to 120 V at 4 °C in a 7.5% separating gel in 1.5 mmol/L Tris-Cl (pH 8.9) until the indicator reached the bottom of the gel. A running buffer (25 mmol/L Tris-Cl, 192 mmol/L glycine, pH 8.3) was used in the trials. After electrophoresis, the gel was incubated at 37 °C in a 20 mL solution containing 4 mL of 5 mg/mL NAD+, 2.5 mL of 0.1 mol/L NaCl, 10 mL of 1 mg/mL nitrobenzene thiocyanate chloride (NBT), 1 mL of 1 mg/mL phenazine methosulfate (PMS), 2.5 mL of 1 mol/L sodium lactate and 5 mL of 0.5 mol/L phosphate buffer (pH 7.5) until dark blue bands appeared. The gels were scanned using a scanner (Hewlett-Packard Development Company, USA). LDH isozymes were quantified using ImageJ 1.48 and expressed as the percentage of M and H subunits (Klebe,

1975). Protein concentrations of samples were measured in duplicate according to the method using bovine serum albumin as the standard (Lowry *et al*., 1951).

2.3. RNA preparation, cDNA synthesis, and expression analysis Total RNA was extracted from tissues (50–100 mg) using RNAiso Plus reagent (Taraka, Japan). The integrity of the RNA was checked using 1.5% agarose gel (Gene Tech, Shanghai, China), and its purity and concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Elimination of residual genomic DNA and reverse transcription of total RNA was performed using a PrimeScript™ RT reagent kit with gDNA Eraser (Taraka, Japan). In detail, a 10 μL reaction volume containing 1 μg RNA, 2 μL 5× gDNA Eraser Buffer, 1 μL gDNA Eraser and RNase-free dH_2O was treated at 42 °C for 2 min to eliminate genomic DNA. To generate the first-strand cDNA, 4 μL of 5× PrimeScript Buffer, 1 μL of PrimeScript RT Enzyme Mix, 1 μL of RT Enzyme, and 4 μL RNase-free dH_2O were added to the total RNA solution, and then treated at 37 °C for 15 min and at 85 °C for 15 s. cDNA was amplified by touch-down PCR with primers designed according to the sequences of *Anolis carolinensis* (Ldh-A accession number: XM_003214746.1; Ldh-B accession number: XM_003220767.1). Primer sequences are shown in Table 1. A Mastercycler Gradient Thermal Cycler (Eppendorf, Germany) was used to amplify partial cDNA in a total volume of 20 μL with 100 ng of cDNA, 2 μL 10× Taq Buffer, 2.4 μL of Mg^{2+} (25 mM), 1 μL of dNTP (20 mM), 0.5 μL of each primer (20 mM) and 0.5 U of Taq DNA polymerase (Sangon Biotech, China). The products were separated on 2% low melting agarose gels, purified with an EZ Spin Column DNA gel extraction kit (Sangon, China), cloned into PMD18-T vector (Takara Bio, Japan), and sequenced (Sangon, China). After obtaining partial sequences of the two genes (Ldh-A and Ldh-B), we designed specific primers for qPCR (Table 1).

Expression patterns of Ldh-A and Ldh-B genes were analyzed using quantitative real- time PCR (qRT-PCR). Real-time PCR experiments were carried out on a CFX-96 real-time PCR system (Bio-Rad, CA, USA). Reactions were run in triplicate with at least three repetitions in a 20 μL volume containing 500 ng of cDNA, 10 μL of 2 SYBR® Premix Ex Taq™ II (Takara Bio, Japan), 0.4 μM of each primer and RNase-free dH_2O. The PCR conditions were: a pre-incubation step (95 °C for 30 s), 40 cycles of amplification (95 °C for 5 s, melting temperature for 30 s and 72 °C for 30 s) and a melting curve (65 °C for 5 s, denaturation from 65 to 95 °C at 0.5 °C/5 s increments) to ensure the specificity of the amplification. The amplification efficiency of genes was measured by the slope of a standard curve as shown in Supplementary Table 1.

For standard curves, triplicate assays were run using serial ten-fold cDNA as templates to PCR-amplify the target and reference genes. Two reference genes, 18S rRNA and ACTB (β-actin), were selected based on expression stability. Fold-changes from *P. erythrurus* to *P. przewalskii* (between treatments and controls) were determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.4. Amplification for full-length cDNA of Ldh-A and Ldh-B To obtain full-length Ldh-A and Ldh-B genes, total RNA was used as template for a rapid amplification of cDNA ends (RACE) procedure. According to the manufacturer's instructions, 5'-RACE-ready cDNA and 3'-RACE-ready cDNA were synthesized using a SMART™ RACE cDNA Amplification Kit (Clontech Laboratories). 5'-RACE and 3'-RACE reactions were performed with the conditions as follows: pre-denaturation at 97 °C for 5 min; denaturation at 97 °C for 30 s, 70 °C for 30 s, reducing by 2 °C every three cycles to Tm and 72 °C for 1 min; 25 cycles at 55 °C for 30 s, Tm for 30 s and 72 °C for 10 min. Primers for RACE PCR are shown in Table 1. The product was separated, purified, cloned, and sequenced as described above.

2.5. Sequence alignment and structure modeling Full-length Ldh-A and Ldh-B cDNA was assembled using DNASTAR Lasergene 7.1. Amino acid sequences were determined using the European Bioinformatics Institute (EBI) database (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). The isoelectric point (pI) and molecular weight (MW) of proteins were predicted using the ProtParam tool (http://web.expasy.org/protparam/). The 3D models of LDHA and LDHB monomeric structure were constructed using Modeller 9.16 software with templates of human proteins (PDB accession number: 1i0z.1) and mouse testicular lactate dehydrogenase C4 (PDB accession number: 2ldx.1). The protein structures were visualized with VMD1.9.2 and Swiss-PDB Viewer (Arnold *et al*., 2006; Kiefer *et al*., 2008; Guex *et al*., 2009; Biasini *et al*., 2014). In addition, hydrogen bonds and pockets differing in volume and shape were predicted and calculated according to the 3D models LDHB monomeric structure (http://cib.cf.ocha.ac.jp/bitool/HBOND/ and http://altair.sci.hokudai.ac.jp/g6/service/pocasa/)

2.6. Statistical analysis All data were tested for normality and homogeneity of variances to meet the assumptions of parametric testing prior to analysis, and

no significant deviations were evident in the data. Data on morphological traits, values of Q_{10} and gene expression were analyzed using ANOVA followed by Tukey's post hoc test. All data were presented as mean ± SEM and were obtained using SPSS release 16.0.0 (SPSS, Inc., Chicago, Illinois, USA). A P-value < 0.05 was considered as a significant difference with a 95 % confidence interval.

3. Results

3.1. LDH activity and lactate concentration

Heart LDH activity was measured for the forward and reverse reactions with pyruvate and lactate as substrate, respectively. The forward reaction activity of LDH in *P. erythrurus* heart was higher than that in *P. przewalskii* at all three temperatures tested (all $F \geqslant 21.434$, $P < 0.001$; Figure 1A). *P. erythrurus* exhibited higher reverse reaction activity of LDH than *P. przewalskii* only at 25 °C and 35 °C ($F \geqslant 5.126$, $P \leqslant 0.038$; Figure 1B) but not at 10 °C ($F_{1,17} = 3.099$, $P = 0.097$; Figure 1B). The thermal sensitivity of enzyme activity was expressed as Q_{10} values, which were calculated from the three temperatures tested (Table 2). Q_{10} values of the forward reaction were significantly lower in *P. erythrurus* (all $F \geqslant 9.556$, $P \leqslant 0.025$). Similarly, *P. erythrurus* has lower Q_{10} values of the reverse reaction than *P. przewalskii* at temperature ranges of 10–25 °C and 10–35 °C (all $F \geqslant 8.191$, $P \leqslant 0.019$). In the heart, lactate concentration of *P. przewalskii* was 0.88 ± 0.12 mg/g protein, 1.35-fold higher than that of *P. erythrurus* ($F_{1,12} = 8.921$, $P = 0.012$; Figure 2).

In skeletal muscle, LDH activities for both forward (all $F \geqslant 5.863$, $P \leqslant 0.039$; Figure 1C) and reverse reactions (all $F \geqslant 5.943$, $P \leqslant 0.041$; Figure 1D) were lower in *P. erythrurus* than that in *P. przewalskii* at all temperatures used. The thermal sensitivity of the LDH forward reaction (all $F \geqslant 4.975$, $P \leqslant 0.044$; Table 2) and the Q_{10} values of the reverse reaction (all $F \geqslant 0.744$, $P \geqslant 0.077$; Table 2) in skeletal muscle had no significant difference between the two species. Moreover, there was no notable variation in lactate concentration of skeletal muscle between *P. erythrurus* and *P. przewalskii* ($F_{1,12} = 1.735$, $P = 0.212$; Figure 2).

3.2. LDH isozyme distribution In heart, the H subunit predominated in both lizards: H subunits accounted for 94.27% ± 0.50% of LDH isoenzymes in *P. erythrurus* and 84.24% ± 0.50% in *P. przewalskii* (all $F = 82.329$, $P < 0.001$; Figure 3B). Moreover, the proportion of M subunits in *P. erythrurus* was 5.73% ± 0.50%, lower

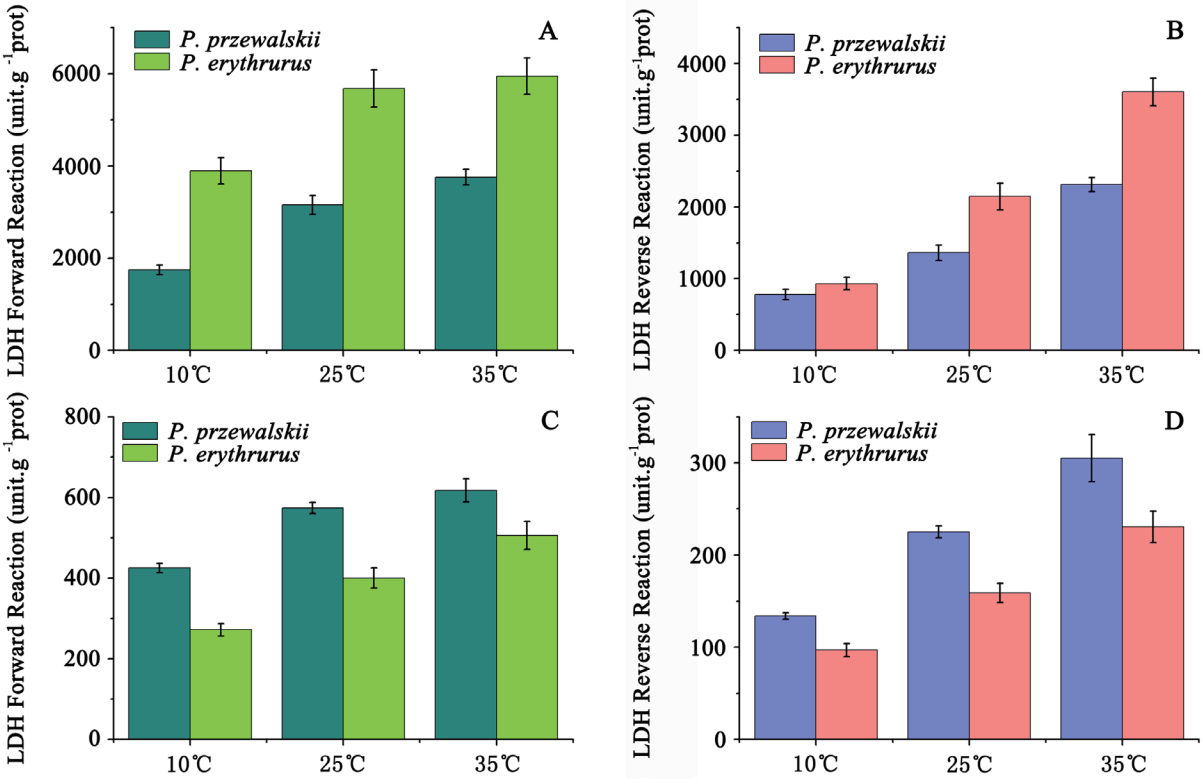

Figure 1 Forward and reverse activity of LDH in heart (A and B) and skeletal muscle (C and D) between *P. erythrurus* and *P. przewalskii* at three assay temperatures. Values are means ± SEM.

Table 1 Meteorological parameters of two sampling sites (Tuotuo River and Jingtai).

Location	Tuotuo River					Jingtai				
Meteorological parameter	Mean temperature (°C)	Highest mean temperature (°C)	Lowest mean temperature (°C)	Atmospheric pressure (Pha)	Sunshine duration (Hour)	Mean temperature (°C)	Highest mean temperature (°C)	Lowest mean temperature (°C)	Atmospheric pressure (Pha)	Sunshine duration (Hour)
Annual Values	–4.2	4.3	–11.3	584.8	8.04	9	15	3	863.9	8.42
January	–16.7	–7	–24.9	579.7	7.06	–8.6	–0.5	–15.1	868.2	7.49
February	–13.6	–4.6	–22	578.8	7	–4.7	3.3	–11.5	866.2	7.7
March	–8.6	0.3	–17.1	580.8	7.4	2.3	10.2	–4.6	864.2	7.94
April	–3.8	4.9	–11.8	583.8	8.56	10.6	18.6	2.8	862.2	8.73
May	1.3	9.4	–6	585.8	8.95	16.8	24.3	8.8	860.8	9.39
June	5.2	12.8	–1	586.5	8.58	21	28.5	13.3	857.8	9.59
July	7.5	14.5	1.7	587.7	8.32	23.2	30.6	15.9	856.5	9.31
Augest	7.2	14.3	1.5	588.7	8.32	21.8	29.1	14.9	859.1	9.06
September	3.5	10.8	–1.7	588.7	7.85	16.1	23.7	9.3	864	8.48
October	–3.9	4.4	–10.2	587.8	8.59	8.2	16.4	1.4	868.1	8.04
November	–12.1	–2.2	–19.9	585.8	8.31	–0.1	7.8	–6.1	869.9	7.89
December	–16	–5.9	–24.1	583.1	7.57	–6.6	1.2	–12.6	869.8	7.4

Table 2 Thermal sensitivity (Q_{10}) for LDHA and LDHB in heart of *P. przewalskii* and *P. erythrurus*. Values are means ± SEM.

Tissue		Temperature range	*P. przewalskii*	*P. erythrurus*	*F* value	*P* value
Heart	Forward	10–25	1.49 ± 0.04	1.29 ± 0.04	9.556	**0.017**
		25–35	1.19 ± 0.02	1.05 ± 0.01	11.296	**0.025**
		10–35	1.36 ± 0.03	1.18 ± 0.03	10.572	**0.018**
	Reverse	10–25	1.45 ± 0.04	1.75 ± 0.09	13.867	**0.008**
		25–35	1.70 ± 0.08	1.68 ± 0.03	0.091	0.768
		10–35	1.55 ± 0.03	1.72 ± 0.05	8.191	**0.019**
Skeletal muscle	Forward	10–25	1.22 ± 0.02	1.30 ± 0.01	4.975	**0.041**
		25–35	1.08 ± 0.03	1.27 ± 0.02	10.884	**0.019**
		10–35	1.16 ± 0.02	1.28 ± 0.03	5.355	**0.044**
	Reverse	10–25	1.42 ± 0.04	1.39 ± 0.09	0.744	0.561
		25–35	1.36 ± 0.03	1.45 ± 0.02	5.943	0.077
		10–35	1.39 ± 0.01	1.42 ± 0.02	2.864	0.581

than that in *P. przewalskii* (15.76% ± 0.50%; Figure 3B). The H/M ratio of *P. erythrurus* heart 17.39 ± 1.85, significantly higher than that of *P. przewalskii* heart (5.49 ± 0.39; $F_{1,13} = 39.816$, $P < 0.001$; Figure 3D).

In skeletal muscle, the M subunit was the major LDH isozyme, with a higher proportion in *P. przewalskii* (96.76% ± 0.69%) compared with in *P.erythrurus* (75.17% ± 1.27%) ($F_{1,14} = 205.533$, $P < 0.001$; Figure 3C). In other words, *P.erythrurus* a higher proportion of H subunits in the skeletal muscle (28.43% ± 1.27%) than its counterpart *P. przewalskii* (3.24% ± 0.69%, Figure 3E).

3.3. Expression of Ldh-A and Ldh-B mRNA in tissues The expression level of Ldh-A in *P. erythrurus* heart was lower than that in *P. przewalskii* whether 18S ($F_{1,9} = 11.979$, $P = 0.009$) or ACTB ($F_{1,9} = 18.393$, $P = 0.003$) was used as the reference gene (Figure 4A). Conversely, Ldh-B expression in *P. erythrurus* heart was higher when 18S was used as the reference gene ($F_{1,8} = 10.365$, $P = 0.015$) but no different when ACTB was used ($F_{1,8} = 1.098$, $P = 0.322$; Figure 4B).

In skeletal muscle, Ldh-A expression in *P. erythrurus* was lower with both reference genes (18S: $F_{1,11} = 4.325$, $P = 0.034$; ACTB: $F_{1,10} = 6.603$, $P = 0.030$; Figure 4C) Ldh-B expression in *P. erythrurus* was higher than in *P. przewalskii* in skeletal muscle with both reference genes (18S: $F_{1,9} = 14.127$, $P = 0.006$; ACTB: $F_{1,10} = 5.839$, $P = 0.039$; Figure 4D).

3.4. Sequence analysisi and structure modeling of LDHA and LDHB The 1 832 bp and 1 819 bp of Ldh-A cDNA (GenBank accession numbers: KX856922 and KX856923), obtained from *P. erythrurus* and *Pprzewalskii*, respectively, both contain a 999 bp of open reading frame (ORF). The deduced amino acid sequences of *P. erythrurus* LDHA and *P. przewalskii* LDHA are identical to each other (Figure 5), and showed sequence identities ranging from 84% (*Mus musculus*) to 98% (*Iguana iguana*) by comparing it with the LDHA sequences from other species (Supplementary Table 2). The predicted MW and theoretical pI of LDHA are 36.57 kDa and 7.72, respectively.

The 1 319 bp of Ldh-B cDNA from *P. erythrurus* (GenBank accession number: KX856924) and 1 302 bp

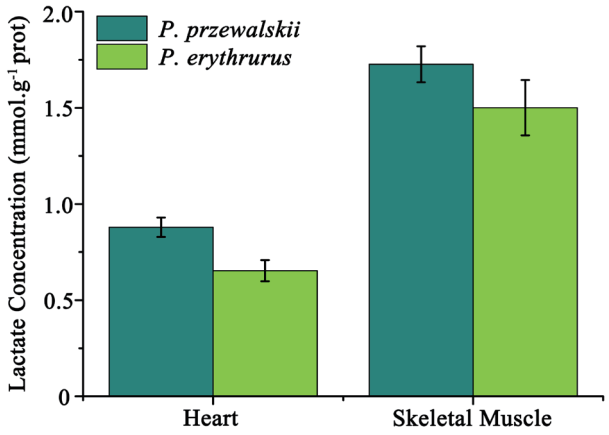

Figure 2 Lactate concentration of heart and skeletal muscle between *P. przewalskii* and *P. erythrurus*. Values are means ± SEM.

of Ldh-B cDNA from *P. przewalskii* (GenBank accession number: KX856924) have a 1 002 bp of ORF encoding 333 amino acid residues. The predicted MW of both LDHB is 36.33 kDa. The pIs of the two LDHB are 7.07 and 6.71, respectively. Three residues were found to differ between the two LDHB by performing an amino acid sequence alignment: Val12Ile, Asn21Ser and Asn318Lys (*P. przewalskii* → *P.erythrurus*) (Figure 6). In addition, the deduced amino acid sequence identities of LDHB between *P. przewalskii/P.erythrurus* and other species range from 86% (*Homo sapiens* and *Mus musculus*) to 95% (*Iguana iguana* and *Anolis carolinensis*) (Supplementary Table 3). The NADH cofactor- binding domain with a Rossmann-type fold formed by residues 22–162 and 250–265 and the mixed α/β substrate-binding domain formed by residues 163–249 and 266– 329 represent the two major domains of LDHA and LDHB. In the structure of LDHB monomer, residue Val12 or Ile12 is on the N-terminal arm and far from the active site. However, both the Val12 and Ile12 of chain A can form a hydrogen bond with Thr301 of chain D and the distance of the two amino acids in *P. erythrurus* and *P. przewalskii* was 1.92 Å and 1.88 Å of the neighboring subunit. Furthermore, residue 21 of *P. erythrurus* was Ser, which can form hydrogen bond with Asp46 of the βB and the distance is 2.77 Å (Figure 8A).However, residue 21 of *P. przewalskii* is Asn, which cannot form hydrogen bond with the 46Asp and the distance is 5.61 Å (Figure 8B). The pockets prediction shows that the volumes of NADH cofactor-binding pockets of *P. erythrurus* and *P. przewalskii* are 107 $Å^3$ and 57$Å^3$, respectively (Figure 8).

4. Discussion

This is the first report to explore the possible adaptation characteristics of LDH in *P. erythrurus*, which is a lizard species inhabiting the highest altitude. Compared to the *P. przewalskii* living at intermediate altitude (1 400 m), *P. erythrurus* exhibited notable differences in LDH activities, thermal sensitivity, isozyme distribution, cDNA sequences, protein structure and tissue-specific expression. These results suggest that compared with previous studies on humans, other mammals and *P. vlangalii* (another lizard living on the Qinghai-Tibet Plateau, ranging from 2 800 to 4 300 meters) living at high altitude, *P. erythrurus* has a strong ability to shift between anaerobic and aerobic metabolism via LDH.

4.1. Forward and reverse reactions of LDH in heart

The heart of *P. erythrurus* has a high LDH activity for the forward reaction, which is contradictory to a common pattern known as "the lactate paradox". Over decades, analysis of LDH activity in some native species living at high altitudes, especially mammals, has suggested that down-regulation of the LDH forward reaction rate is a common physiological response to hypoxic conditions ("the lactate paradox"; West, 1986; Hochachka, 1988). The response may protect organisms from damage by means of lactate accumulation (Constable *et al*., 1987; Howald *et al*., 1990; Terrados *et al*., 1990; Sheafor, 2003, Qi *et al*., 2008; Ma *et al*., 2012; Tang *et al*., 2013). *P. erythrurus* hearts present an opposite response. Similar results have been found in cardiac muscle of *Ochotona princeps*, a native mammal at high altitude (Sheafor, 2003). These results suggest that the lactate paradox is not a required modification for success at high altitudes.

The higher forward reaction rate in cardiac muscle of *P. erythrurus* may indicate that it has a strong capacity of glycolysis, although its heart may not be dominated by anaerobic metabolism, given that efficient synthesis of ATP in the heart, it is critical to ensure continuous activity. Instead, relatively high citrate synthase activity in its cardiac muscle (10 °C: *P. przewalskii* 0.51 ± 0.09units·g^{-1}, *P. erythrurus* 0.95 ± 0.08 units·g^{-1}, 35 °C: *P. przewalskii* 2.14 ± 0.08 units·g^{-1}, *P. erythrurus* 3.50 ± 0.29 units·g^{-1}, our unpublished data) suggests that aerobic metabolism is probably the main source of energy supply, despite its survival in a hypoxic environment. Similar results were also found in two populations of *P. vlangalii*. LDH activity in cardiac muscle of the lizard at high altitude was significantly lower than that of the lizard at low altitude, but the succinate dehydrogenase (SDH) activity, which is an indicator of aerobic metabolism level, was relatively lower in the lizard at high altitude(He *et al*., 2013). The high aerobic metabolism of *P. erythrurus* heart also indirectly suggests that oxygen supply is sufficient to meet

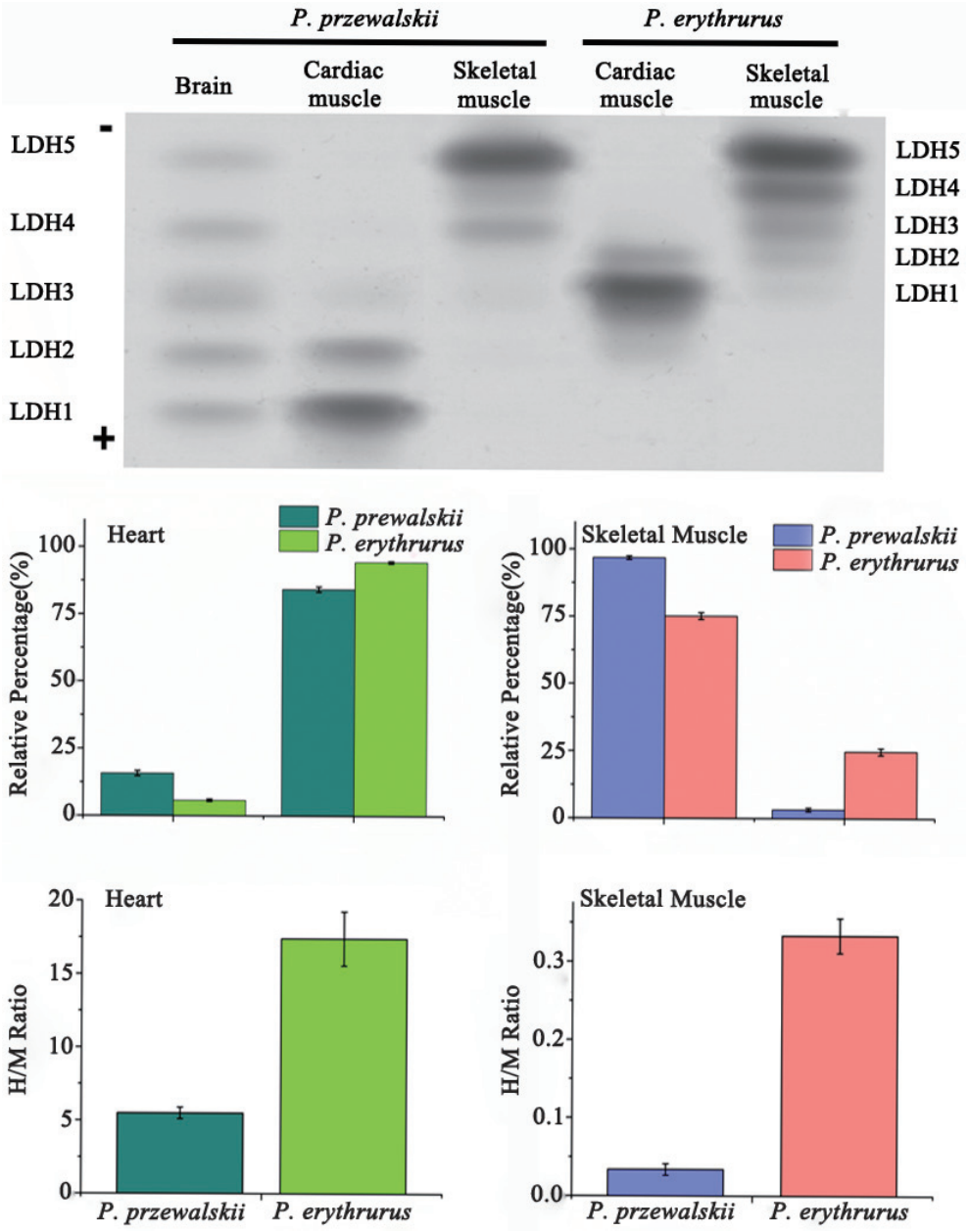

Figure 3 Distribution of the different LDH isoenzymes in gel electrophoresis (A), and quantification of LDH isoenzymes (B and C) and H/M ratio (D and E) in heart and skeletal muscle between *P. przewalskii* and *P. erythrurus*. Values are means ± SEM.

the need of its low metabolic rate (usually less than 20% of the metabolic rate of mammals of equivalent weight) (Brand *et al*., 1991), despite the low concentrations of O_2 at high altitudes. Additionally, our previous study has demonstrated that *P. erythrurus* has higher gene expression levels of myoglobin and concentration in cardiac and skeletal muscle than *P. przewalskii*, indicating species living at high altitudes have a greater oxygen storage and capacity (Xin *et al*., 2015).

The maintenance of a high anaerobic metabolism in *P. erythrurus* heart may be dictated by their habitat factors. The heart and circulatory system are especially sensitive to hypoxia, so effective anaerobic metabolism in the heart would contribute to the maintainance of energy homeostasis of the heart under hypoxic conditions. Furthermore, a change of environmental temperature can also affect the enzyme activity. This adjustment is more important for reptile species that live in a high-altitude habitat and experience a wider fluctuation of environmental temperature (Tang *et al*., 2013). A previous study showed that aerobic capacity decreased significantly during low temperature (4 °C) or hibernation

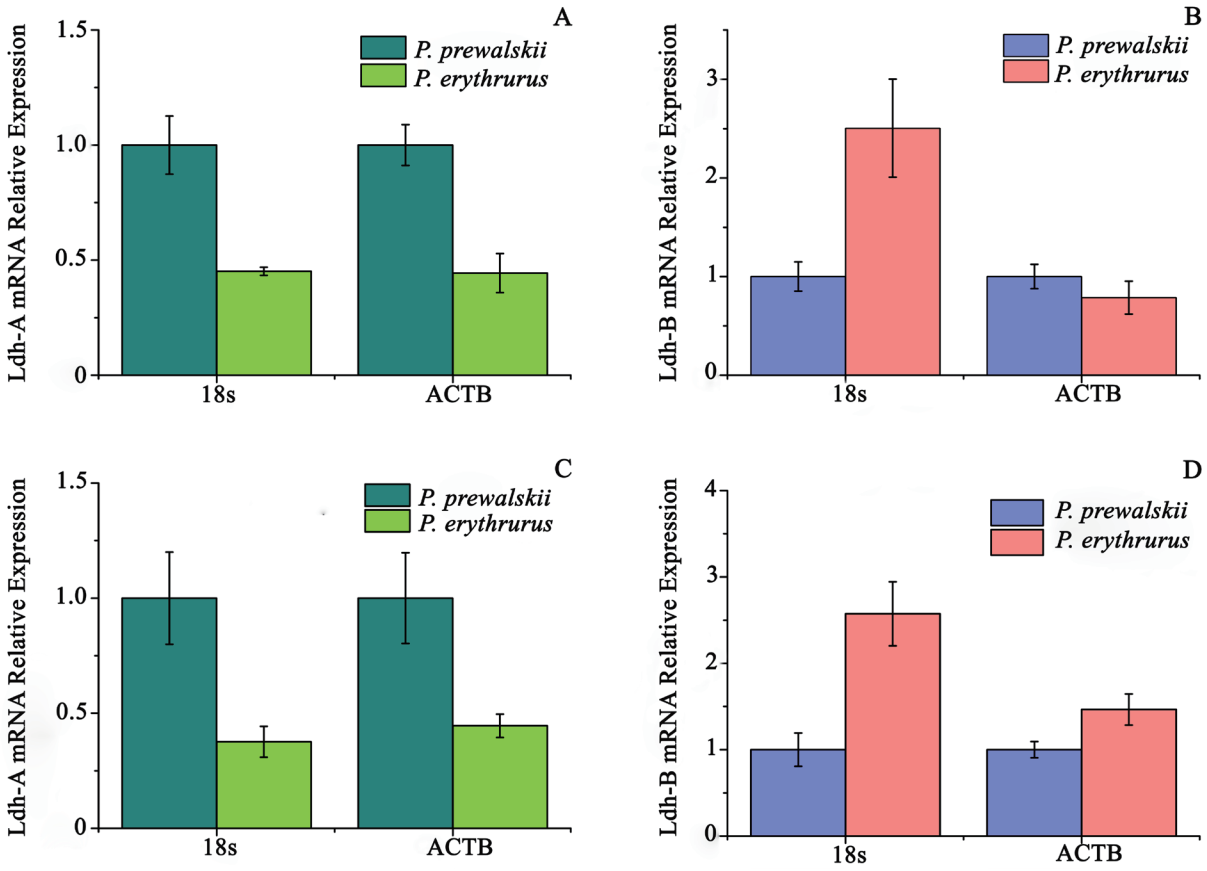

Figure 4 Relative quantifications of the Ldh-A and Ldh-B mRNA in heart and skeletal muscle with different reference genes between *P. erythrurus* and *P. przewalskii*. Values are means ± SEM.

in *P. erythrurus*, but there was no change in the forward reaction of LDH before and after hibernation (Li *et al*., 2017). It is likely that the high activity of LDH forward reaction in *P. erythrurus* heart could potentially contribute to the energy supply at low oxygen partial pressure in the cardiovascular system, or at low temperature in their burrow. However, a further investigation is needed to understand the mechanism of how for anaerobic and aerobic metabolism switches in cardiac muscle under different conditions.

The reverse reaction rates of LDH in *P. erythrurus* heart were also significantly higher than those in *P. przewalskii*. Combined with the lower lactate concentration and higher H:/M ratio found in *P. erythrurus* heart, the high reverse reaction rate of LDH likely presents a strong ability to remove lactate. The heart of *P. erythrurus* can take advantage of lactate as a metabolic substrate, generating pyruvate that can be utilized in other metabolic pathways.

The thermal response, indicated as Q_{10} value, was significantly lower for the forward reaction of *P. erythrurus*. The results demonstrate that *P. erythrurus*, living at high altitude with fluctuating temperature, still has a strong ability to catalyze the conversion of pyruvic acid to lactic acid, with a concomitant release of ATP. This feature is conducive to retaining relatively stable locomotion ability under fluctuating environmental temperatures, especially benefitting temperature regulation, reproduction and predator evasion at lower ambient temperatures. It also could be a strategy for *P. erythrurus* to adapt to the plateau's low temperature and low oxygen environment. On the contrary, thermal sensitivity of the reverse reaction in *P. erythrurus* was much higher, especially in the low temperature range (10–25 °C). It suggests that *P. erythrurus* has a stronger ability to catalyze the conversion of lactic acid to pyruvate at relatively high body temperature, making it quickly remove lactic acid and maintain the body's homeostasis. In the current study, the two species both had a predominant distribution of H subunits in the heart, and LDH showed different electrophoretic mobility, which may correlate with the pI and structure of the gene (Markert *et al*., 1975). Meanwhile, *P. erythrurus* had a greater proportion of H subunits, a higher level of Ldh-B expression and higher H/M ratio. The shift of isoenzyme

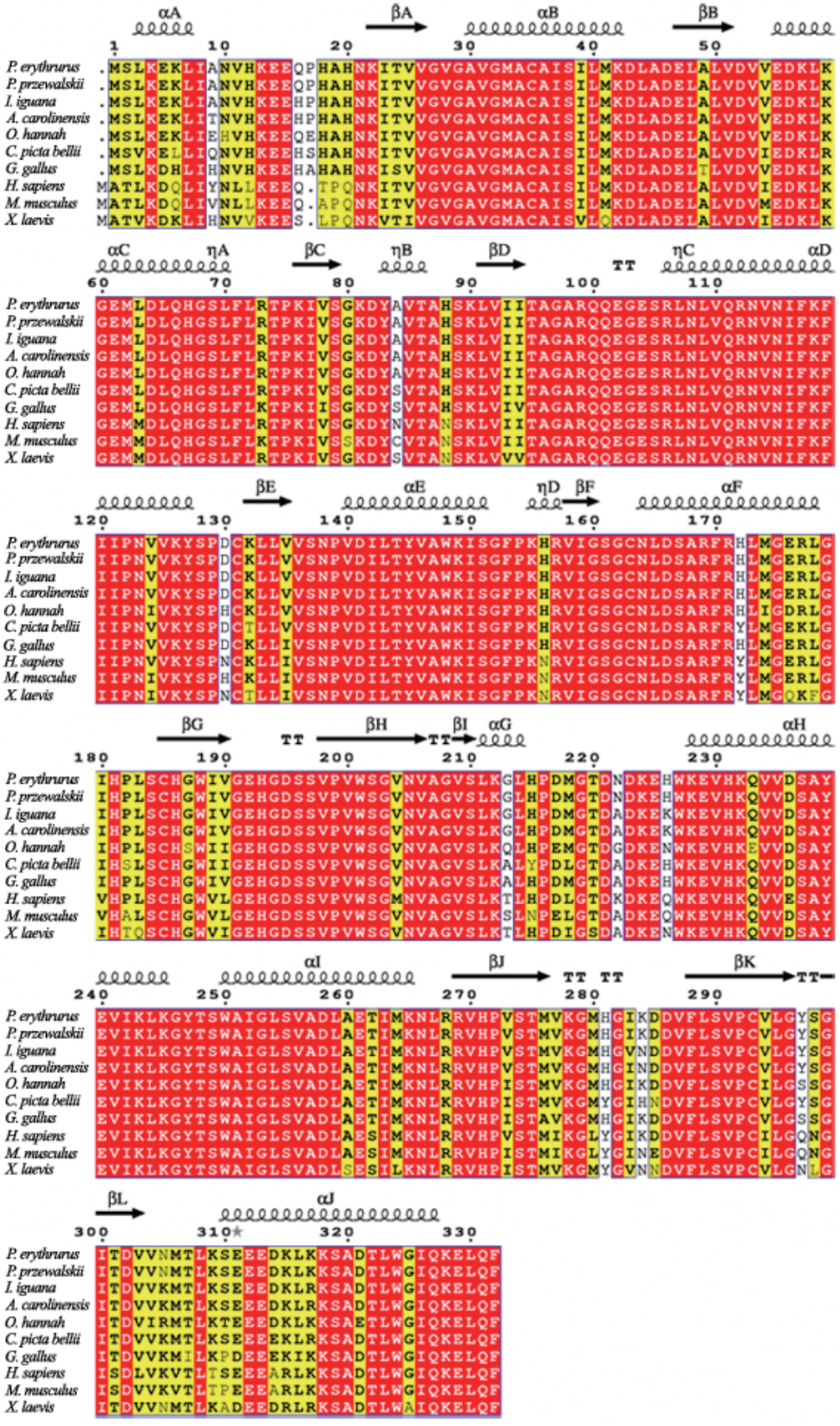

Figure 5 Multiple sequence alignment of LDH-A amino acid sequences from different species. *Iguana iguana* (ABI21884.1), *Python bivittatus* (XP_007435027.1), *Amblyrhynchus cristatus* (ABI21883.1), *Gallus gallus* (NP_990615.1), *Chrysemys picta bellii* (XP_005294003.1), *Homo sapiens* (AAH67223.1), *Mus musculus* (AAA21466.1), *Xenopus laevis* (NP_001080702.1), *Ophiophagus hannah* (ETE69616.1). Conservative amino acids are shaded by red. α, η and β represent Helix, 3/10 helix and beta sheet, respectively.

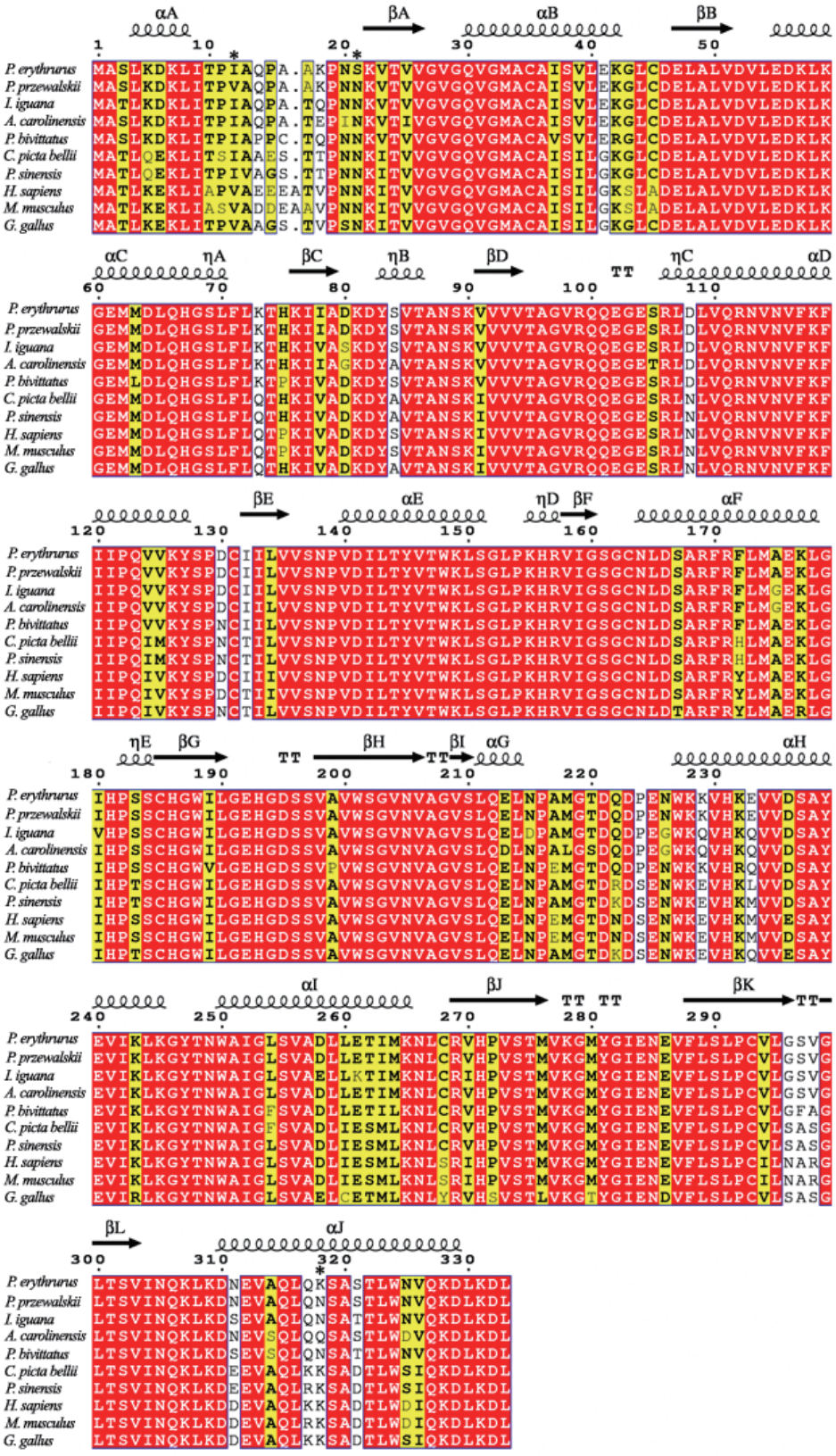

Figure 6 Multiple sequence alignment of LDH-B amino acid sequences from different species. *Iguana iguana* (AAN05099.1), *Anolis carolinensis* (XP_008108535.1), *Python bivittatus* (XP_007427442.1), *Chrysemys picta bellii* (XP_005296688.1), *Pelodiscus sinensis* (XP_006127717.1), *Mus musculus* (NP_032518.1), *Homo Sapiens* (NP_002291.1), *Gallus gallus* (NP_989508.1). Conservative amino acids are shaded by red and asterisks represent substitution sites between *P. erythrurus* and *P. przewalskii*. α: apha Helix; η:3/10 helix; β: beta sheet.

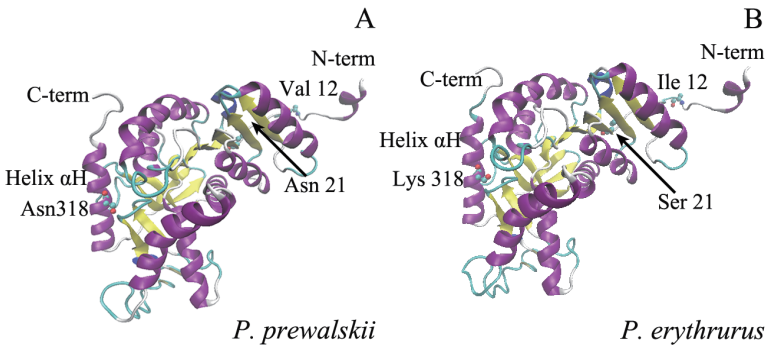

Figure 7 Three-dimensional model of LDH-B from the two species. Three-dimensional model of LDH-B monomer from *P. przewalskii* (A) and *P. erythrurus* (B). The amino acid substitutions between high altitude *P. erythrurus* and low altitude *P. przewalskii* arehighlight. The model was created with Modeller 9.16 using mouse testicular lactate dehydrogenase C4 (PDB accession number: 2ldx.1) as templates.

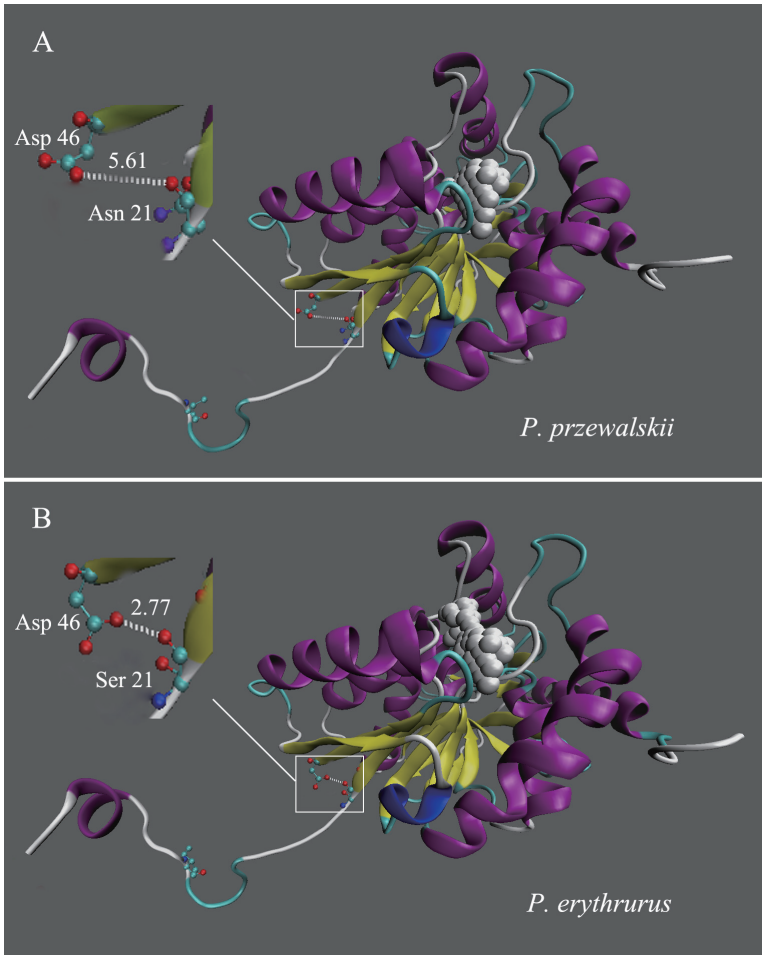

Figure 8 The hydrogen bond and pocket volumn in *P. erythrurus* (A) and *P. przewalskii* (B). Ser21 of *P. erythrurus* can form hydrogen bond with Asp46 of the βB and the distance of hydrogen bond is 2.77. However, Asn21 of *P. przewalskii* cannot form hydrogen bond with the Asp46 and the distance is 5.61 Å; The NADH cofactor-binding pocket volume in *P. erythrurus* and *P. przewalskii* was 107 and 57Å3, respectively.

composition in *P. erythrurus* indicates a greater capacity of lactate oxidation and inhibition of lactate accumulation. During hypoxia and moderate intensity exercise, lactate released from working muscle and other tissue beds is the primary fuel source for the heart, exiting the muscles through MCT (Stanley *et al*., 1988) in a process called the lactate shuttle (Brooks, 1987). Heart tissue typically utilizes lactate as a substrate for the mitochondrial TCA cycle, and its oxidation can produce a significant amount of ATP (Schurr, 2006). The high level of H subunits in

the heart may facilitate re-oxidation of lactate to pyruvate at the site of production, and help to rapidly remove the lactate accumulated during locomotion. A similar result was reported in heart of high-altitude plateau zokor and plateau pika when compared with Sprague Dawley rats (Qi *et al*., 2008). The lower lactate content of *P. erythrurus* supports this inference.

4.2. Forward and reverse reaction of LDH in skeletal muscle Unlike cardiac muscle, skeletal muscle can be rested and rely to a greater extent on the smaller amount of ATP produced by the glycolytic pathway for a portion of its energy production (Sheafor, 2003). The activity of LDH in skeletal muscle was completely opposite to that in the heart. Both forward and reverse reaction rates of LDH in *P. erythrurus* were significantly lower than that in *P. przewalskii*, following the lactate paradox pattern. These results are likely related to the relatively weak locomotor ability of *P. erythrurus*, especially its sprint speed. A locomotion study showed that the maximum distance moved and the sprint speed of *P. erythrurus* were significantly lower than *P. przewalskii* (unpublished). Different fiber-type compositions of skeletal muscle could be responsible for the correlation between locomotion ability and LDH activity (Gleeson and Harrison, 1986, 1988).

There were also interesting findings from analysis of thermal sensitivity. The Q_{10} value of the forward reaction of *P. erythrurus* was comparable to *P. przewalskii* at 10–25 °C, but much lower than *P. przewalskii* at 25–35 °C. These results indicate that the forward reaction rate of LDH in *P. erythrurus* was lower with small variation but increased significantly at higher temperatures. These characteristics of anaerobic glycolysis in skeletal muscle of *P. erythrurus* suggest that it may have more ATP supply during locomotion and improved sprint speed. In turn, a higher forward reaction rate of LDH in skeletal muscle of *P. przewalskii* with small temperature fluctuation may help it maintain strong locomotion ability, and thus enhance survival at 10–35 °C.

The relative content of H subunits in the skeletal muscle of *P. erythrurus* was significantly higher than that of *P. przewalskii*, although the absolute content of M subunits in both lizards was higher. This result indicates that *P.erythrurus* can remove lactate more efficiently in skeletal muscle, consequently reducing the time for which it is required to remain inactive while lactate is converted to pyruvate (Sheafor, 2003). It also can compensate for its relatively low reverse reaction activity of LDH and provide a benefit by maintaining homeostasis of the intracellular environment in skeletal muscle (Hochachka and Mommsen, 1983). Furthermore, high levels of H subunits in *P. erythrurus* skeletal muscle can facilitate the reconversion of lactate to pyruvate at the site of production and reduce the amount of lactate transported to the heart or other tissues via the circulatory system.

4.3. Sequence differences and their location in the three-dimensional structure As shown in the structure model of the LDH monomer, the helix αH and two helices α1G and 2G in the substrate-binding domains form the margins through which cofactor and substrate enter the active site before the catalytic loop closes. These structures have been described to be important for catalysis in many species (Fields and Houseman, 2004; Fields *et al.*, 2008; Johns and Somero, 2004). Therefore, introduction of mutations in the helices may have the potential to affect the function of the LDH enzyme. Amino acids alignment reveals that three residues, located at the N-terminus and helixαH, are different between *P.erythrurus* LDHB and *P. przewalskii* LDHB.

Among these mutations, residue 12 can adopt an extended conformation and wrap around the adjacent subunit in the tetramer of the LDH (Grau *et al*., 1981; Read *et al*., 2001). The relatively short distance between residue 12 of A chain and residue 301 of D chain in *P. erythrurus* can increase stability of the LDH enzyme. Similarly, the substitution of K7R in N-terminal regions of LDHB contributes to the stability of the protein and plays an important role in adaptation to environment pressures (Brindley *et al*., 2008). In addition, together with the substitution I283V, the substitution of T9A can make an *A. cristatus* ortholog adapt to a cold environment by decreasing the Km and Kcat values of LDHB for pyruvate. It may also help to form a hydrogen bond with the side chain amino group of 305K to stabilize the loop region leading to helix αH (Fields *et al*., 2008). In our result, the residue at position 12 is visualized as interacting with residues on strand βM from a second monomer, which anchors an extended strand leading to helix αH and has an effect on controlling the position and movement of αH. Furthermore, the substitution V12I results in the addition of an extra methylene group. A number of studies have shown that increasing residue size, hydrophobicity and packing efficiency can enhance protein stability (Haney *et al*., 1999). Compared with the Val12 in *P. przewalskii*, the Ile12 in *P. erythrurus* may increase protein stability, decrease mobility and thus increase substrate affinity and enzyme activity. Residue 21 of *P. erythrurus* is Ser, which can form a hydrogen bond with Asp46 of the βB and the distance of hydrogen bond is 2.77 Å. However, the Residue Asn21

of *P.przewalskii* cannot form a hydrogen bond with the 46Asp and the distance is 5.61 Å. Therefore, substitution N21S of *P. erythrurus* can strengthen the rigidity of protein. Moreover, Residue 21 and 45 are involved in the formation of NAD(H) binding active pockets, the increase of rigidity makes the volume of the active pockets become larger in *P. erythrurus* (107) than *P. przewalskii* (57) (Figure 8), which makes it easier for NADH to enter.

In conclusion, *P. erythrurus* presented increased anaerobic potential and greater ability to oxidize lactate acid in the heart when compared with its counterpart *P. przewalskii*, but the results were quite different in skeletal muscle. These adaptations may involve in the modification of isoenzyme composition, mRNA expression and protein structure. However, a further investigation is necessary to confirm this in future.

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Appendix

Table S1 Primer sequences for RT-PCR amplification, gene expression analysis and RACE PCR.

Primer name	Sequence (5'-3')	Product size/bp	Melting temperature/°C
Touch-down PCR primers			
LdhA-F	CCTGGTTCAGCGCAATGTGAACA	518	
LdhA-R	GCATGCCCTTGACCATTGTAGAA		
LdhB-F	GCAGCGGCTGCAACCTTGACTCT	432	
LdhB-R	TCACACTGGTCAAGCCGACAGAC		
Real-time PCR primers			
LdhA-QF	CCTTTCTGTGGCCGATTTAGC	132	57.2
LdhA-QR	GAGTAGCCCAGCACACAAGG		
LdhB-QF	GGTTGACAGTGCCTATGAAG	115	57.2
LdhB-QR	CGGATGAACTCGGCAAAGG		
ACTB-QF	CCCATTGAGCACGGCATT	146	57.2
ACTB-QR	CTTTTCCCTGTTGGCTTTGG		
18S-QF	AGACGAACCAGAGCGAAAGCA	122	59.1
18S-QR	GATCGCTAGTCGGCATCGTTT		
5’ RACE PCR primers			
LdhA-5R1	GAGTAGCCCAGCACACAAGG		
LdhA-5R2(nested)	GCATGCCCTTGACCATTGTAGAA		
LdhB-5R1	TCACACTGGTCAAGCCGACAGAC		
LdhB-5R2(nested)	CGGATGAACTCGGCAAAGG		
3’ RACE PCR primers			
LdhA-3F	CCTGGTTCAGCGCAATGTGAACA		
LdhB-3F	GCAGCGGCTGCAACCTTGACTCT		

Table S2 Amplification efficiencies of target and reference genes.

Gene name	Amplification efficiency (%)	R^2
Ldh-A	97.3	0.998
Ldh-B	96	1
ACTB	96.9	0.999
18S	96.9	0.999

Table S3 Multiple sequence alignment of LDH-A amino acid sequences from different species.

Species	Genbank ID	Similarity (%)
Iguana iguana	ABI21884.1	95
Python bivittatus	XP_007435027.1	95
Amblyrhynchus cristatus	ABI21883.1	98
Gallus gallus	NP_990615.1	93
Chrysemys picta bellii	XP_005294003.1	92
Homo sapiens	AAH67223.1	87
Mus musculus	AAA21466.1	87
Xenopus laevis	NP_001080702.1	84
Ophiophagus Hannah	ETE69616.1	94

Table S4 Multiple sequence alignment of LDH-B amino acid sequences from different species.

Species	Genbank ID	Pe Similarity (%)	Pp Similarity (%)
Iguana iguana	AAN05099.1	95	95
Anolis carolinensis	XP_008108535.1	95	95
Python bivittatus	XP_007427442.1	93	93
Chrysemys picta bellii	XP_005296688.1	88	87
Pelodiscus sinensis	XP_006127717.1	88	88
Mus musculus	NP_032518.1	87	87
Homo Sapiens	NP_002291.1	87	87
Gallus gallus	NP_989508.1	86	86