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Source: Journal of Vertebrate Biology, 72(23008)

Published By: Institute of Vertebrate Biology, Czech Academy of Sciences

URL: <https://doi.org/10.25225/jvb.23008>

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Phenotypic trait variations in the frog *Nanorana parkeri*: differing adaptive strategies to altitude between sexes

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► Received 18 January 2023; Accepted 13 March 2023; Published online 10 April 2023

Abstract. In many animals, changes in altitude drive adaptive variation in body size. However, how other phenotypic traits change when faced with different environments has been little studied in ectotherms. In this study, we selected the high Himalaya frog *Nanorana parkeri* as a model species for investigating the adaptive evolution of phenotypic traits that respond to altitude in both sexes. First, we found that body mass in populations at higher altitudes was lower than at lower altitudes in females, with no difference observed in males. Second, we found significant differences in fresh liver mass, fresh heart mass, and the ratio of liver mass to body mass with increasing altitude, while hindlimb length decreased with altitude in both sexes. Third, snout-urostyle length, hindlimb length, fresh heart mass and the ratio of heart mass to body mass showed significant negative correlations with increasing altitude in both sexes. In contrast, body mass showed a significant correlation with altitude in females but not males. On the other hand, the ratio of liver mass to body mass showed a significant correlation with altitude in males but not in females. Thus, the species displayed sex-specific organ-size variation along elevation gradients, which may trade-off in life history strategies among populations. We speculate that selection favours a larger heart and liver mass to maintain a higher respiratory rate and energy consumption as an adaptation to high-altitude environments.

Keywords: high Himalaya frog, adaptation, oxygen supply, energy requirement, organ size

Introduction

Local adaptation of animal life histories, such as geographical body size variation, have been widely

observed (Bernardo 1994, Klaassen 1995, Morrison & Hero 2003, Valenzuela-Sanchez et al. 2015). However, the underlying physiological and morphological correlates of this variation have been little studied. For

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example, organ size could potentially be associated with geographic life-history variation (Liao et al. 2016, Oromi et al. 2016), while metabolism, rates of biosynthesis and high somatic tissue investment may affect the life expectancy and trade-off between self-survival and reproduction investment of animals living in resource-limited environments (Hilton et al. 2000). It is also possible that correlated organs of animals in marginal environments could increase in size to meet increased energy demands (Daugirdas et al. 2008, Zhong et al. 2017). The nature and magnitude of organ size adaptations against different variables (e.g. latitude, altitude, temperature and precipitation) and the resultant trade-offs with other aspects of organism function may be the underlying factors in determining the distributional limits of wild animals (Mai et al. 2019).

Within different species, geographically separated populations experience different local environments and, consequently, different selective pressures (Morrison & Hero 2003, Dammhahn et al. 2018). In many species, especially those having broadly altitudinal and/or latitudinal distributions, different geographical populations are likely to exhibit geographical variations in morphology and physiology, showing adaptive evolution of phenotypic traits to local ecological conditions (Morrison & Hero 2003). In ectotherms, body size has been shown to display adaptive evolution to local climates (Zhang et al. 2012); however, the underlying mechanisms of such changes, such as body composition, organ size and other physiological traits, are rarely studied (Ma et al. 2009, Zhang et al. 2012). Geographical variation in organ size may be an essential means by which animals optimise exploitation of their local environment and could evolve into different adaptive strategies among geographically separated populations (Hilton et al. 2000, Piersma & Drent 2003, Zhong et al. 2017, Mai et al. 2019, Zhao et al. 2019).

At different points in their life history, some animals can adjust the mass of some organs and/or their body composition (Hilton et al. 2000, Piersma & Drent 2003, Zhao et al. 2019). Where energy for growth and development is limited, for example, animals may reallocate the available resources to survival and breeding, with the strategy used depending on the environment in which it lives (Cooke et al. 2016). In unfavourable environments, organs responsible for energy conversion, such as the liver, can be expected to be larger than in less harsh habitats to meet energy demands, which explains the altitudinal increase in liver size in ectotherms (Zhong et al. 2017, Mai et al.

2019, Zhao et al. 2019). Likewise, studies have shown that the morphology and relative size of the heart, the main organ of the circulatory system responsible for pumping blood through the circulatory vessels, is related not only to the organism's physiological traits but also to conditions in the local environment. Consequently, in some species, such as Anurans, relative heart size tends to increase with increasing altitude as a larger heart utilises less oxygen more efficiently (Zhong et al. 2017).

The high Himalaya frog *Nanorana parkeri* is a species endemic to the Tibetan Plateau occurring across a narrow latitudinal but extensive altitudinal range, making it the highest-distributed amphibian in the world (Ma & Lu 2009). Different populations of the species have been found to exhibit different life histories, with females growing slower and living longer than males, along with sexual dimorphism in body size with increasing altitude, with females being larger than males and females showing greater differences in body size than males with increased altitude (Ma et al. 2009, Zhang et al. 2012). The reproductive organs of females and males also show different annual cycles, with females capable of breeding biannually (or more often), while males breed annually (Ma & Lu 2009). While previous studies have explored the adaptation of body size in this frog to altitude over a restricted range (Ma & Lu 2009, Zhang et al. 2012), there is little or no information on adaptive variations in other phenotypic traits.

To better understand how altitude affects the evolution of phenotypic traits, therefore, we sampled *N. parkeri* from a broader range of localities within its natural distribution area than in previous works (Ma et al. 2009, Zhang et al. 2012) and determined a range of morphological traits for both sexes to analyse geographical variations in phenotypic traits between different populations. We hypothesise that the different roles of each sex in breeding, alongside the harsher environment at high altitudes, will impose strong selective pressures on *N. parkeri* survival. Consequently, we predict that the frogs will exhibit geographical variations in morphological traits such as snout-urostyle length, body mass and the ratio of hindlimb length to snout-urostyle length and that physiological traits will show both geographical variations and differing trends between the sexes, with male frogs at higher altitude having relative larger livers and smaller hearts than lower altitude males, and females from higher altitudes having relative smaller livers and larger hearts than lower altitude populations.

Material and Methods

Biological information

Nanorana parkeri are known from southern and eastern Xizang in China and the Tibetan Plateau of Nepal, occurring at 2,850-5,000 m a.s.l. (Jiang et al. 2018). Depending on altitude, the frogs emerge from hibernation between early April and early May. The breeding season lasts approximately three months, during which time the frogs remain active from afternoon to midnight when air temperatures are still relatively high. Spawning occurs in marshy ponds (Zhang et al. 2012) that exhibit altitudinal differences in substrate, pH, water temperature, water depth, vegetation coverage, and fish presence (Fan & Pan 2014).

Animal collection

We sampled 443 male and 173 female frogs from 25 localities on the Tibetan Plateau between May 7 and

July 23, 2017 (Table 1). Frogs were caught by hand during the daytime when they were more active, and sex was determined using the methods of Zhang et al. (2012). Females with inflated abdomens (i.e. carrying eggs) were released immediately after capture, while those with flabby ventral skin (i.e. post-oviposition) were included. Males with nuptial pads on the first digits of the forelimb and clusters of spines on the chest were also included in the study, while non-sexually mature frogs were released back into the spawning ponds immediately after capture. At each sampling site, we noted the altitude, longitude and altitude.

Sampling for this study was approved by the local government body at each sampling location. In addition, the research was approved by the animal welfare committee of Yunnan Normal University (code 20160802).

Table 1. Location, altitude and number of samples of *Nanorana parkeri*.

Sample site	Longitude	Altitude	Altitude (m a. s. l.)	Sample size ♂	Sample size ♀	Total sample size
Basu	E 97°16'	N 30°13'	3,254	20	2	22
Leiwuqi	E 95°22'	N 31°29'	4,283	23	0	23
Changdu	E 96°35'	N 31°15'	4,302	10	10	20
Lasa	E 91°06'	N 29°55'	3,854	10	7	17
Dangxiong	E 91°04'	N 30°28'	4,259	24	23	47
Milin	E 94°21'	N 29°19'	2,927	38	3	41
Gongbujiangda	E 93°39'	N 29°09'	2,993	8	6	14
Linshi	E 94°44'	N 29°42'	3,418	23	4	27
Naqu	E 92°58'	N 31°52'	4,370	21	8	29
Jiali	E 94°42'	N 31°52'	4,402	11	3	14
Nierong	E 90°53'	N 30°42'	4,728	10	1	11
Rikaze	E 89°05'	N 29°34'	3,927	14	1	15
Lazi	E 87°42'	N 29°03'	4,001	5	11	16
Kanmax	E 89°36'	N 28°54'	4,020	10	8	18
Nanmulin	E 87°07'	N 28°26'	4,263	12	18	30
Jiangzi	E 89°09'	N 27°47'	4,334	28	2	30
Niemula	E 86°02'	N 28°12'	4,468	6	1	7
Dingri	E 86°31'	N 29°28'	4,637	15	10	25
Zhongba	E 84°18'	N 29°38'	4,643	13	11	24
Pulan	E 91°37'	N 29°48'	3,766	38	22	60
Qiongjie	E 91°41'	N 29°01'	3,799	21	9	30
Qusong	E 92°08'	N 29°07'	3,825	30	0	30
Cuomei	E 91°55'	N 28°55'	3,974	30	0	30
Sangri	E 92°15'	N 29°42'	4,391	4	2	6
Langkazi	E 90°23'	N 29°21'	4,457	19	11	30

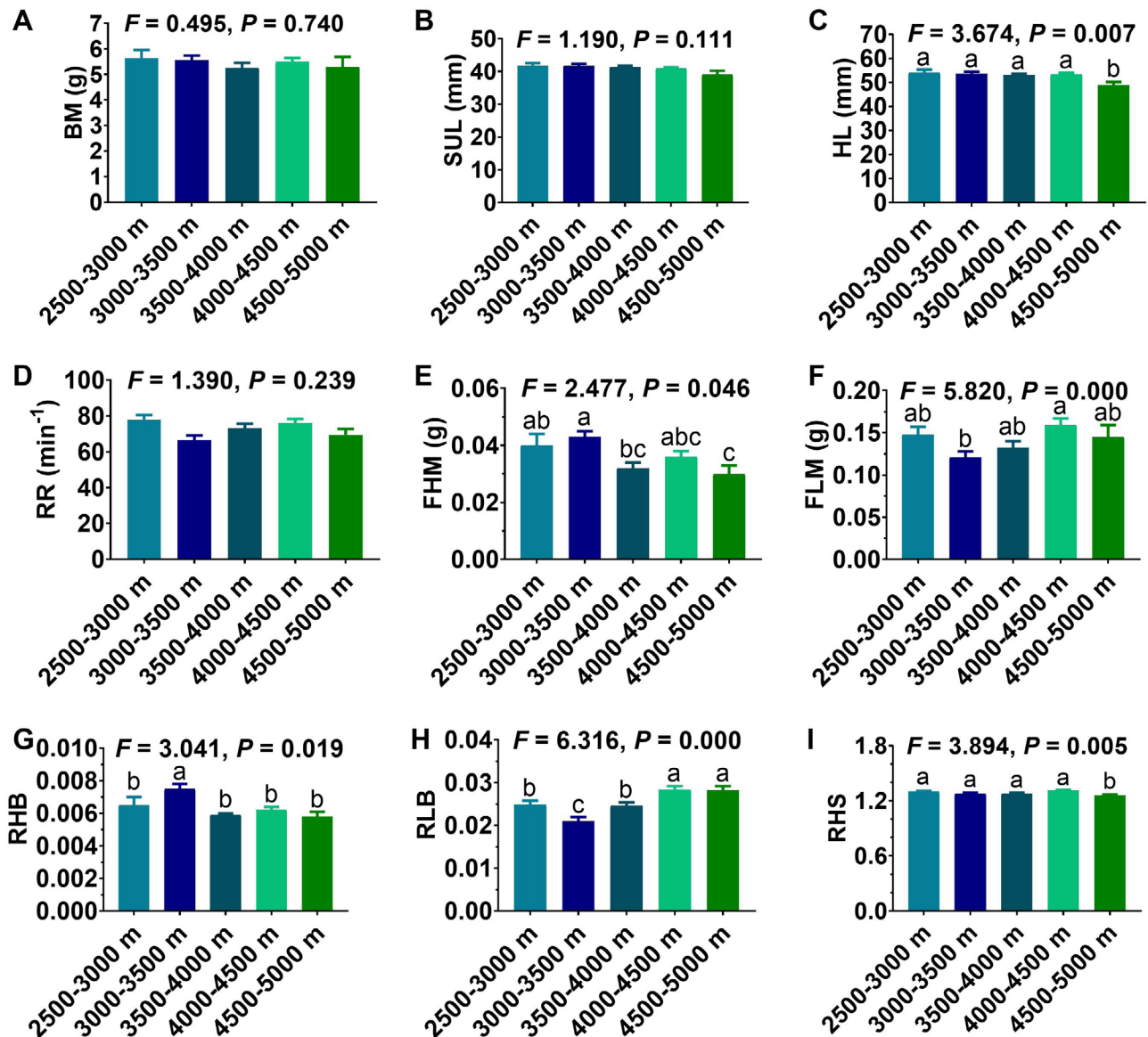


Fig. 1. Comparative analysis of phenotypical traits in male *Nanorana parkeri* among different altitudes. (A) BM: Body mass, (B) SUL: Snout-urostyle length, (C) HL: Hindlimb length, (D) RR: Respiratory rate, (E) FHM: Fresh heart mass, (F) FLM: Fresh liver mass, (G) RHB: Ratio of heart mass to body mass, (H) RLB: Ratio of liver mass to body mass, (I) RHS: Ratio of hindlimb length to SUL. Data were analysed using one-way ANOVA followed by the LSD post hoc test. Significant differences among altitudinal groups were indicated by different alphabetic letters.

Index determination

For each frog, a series of morphological traits were examined, including body mass (BM), body size (snout-urostyle length; SUL), hindlimb length (HL) and the ratio of HL to SUL (RHS), and physiological traits, including respiratory rate (RR), fresh heart mass (FHM), fresh liver mass (FLM), the ratio of FHM to BM (RHB) and the ratio of FLM to BM (RLB).

Immediately after capture, each frog was wiped with degreasing cotton to remove excess water and weighed for BM (nearest 0.01 g) using an electronic balance. Next, the SUL and left HL were measured (nearest 0.01 mm) using an electronic calliper. The frogs were then placed into an open-mouthed

transparent bottle and kept at resting status for 30 min. The oropharyngeal respiration rate per minute was then calculated by monitoring fluctuations in the throat and recalculating this as fluctuations per hour for further analysis. Finally, the frogs were humanely euthanised and dissected by cutting open the chest (male: female = 194:84). The liver and heart were then dried with an absorbent cotton ball, and FLM and FHM measured (nearest 0.01 g) using an electronic balance. Finally, the data were used to calculate RHB, RLB and RHS.

Statistical analysis

Frogs were classified as having been caught in one of five altitude ranges, i.e. 2,500-3,000 m, 3,000-

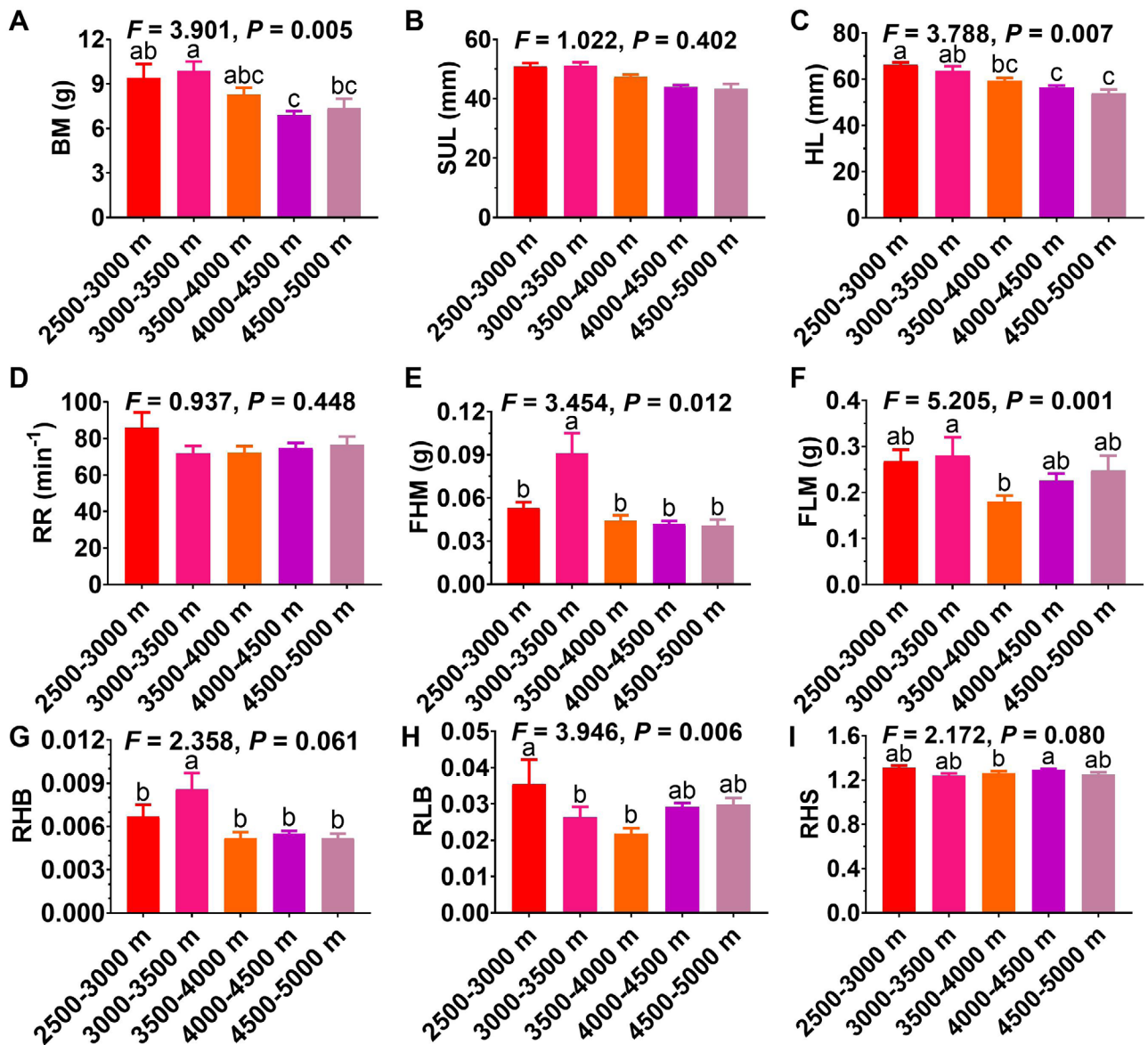


Fig. 2. Comparative analysis of phenotypical traits in female *Nanorana parkeri* among different altitudes. (A) BM: Body mass, (B) SUL: Snout-urostyle length, (C) HL: Hindlimb length, (D) RR: Respiratory rate, (E) FHM: Fresh heart mass, (F) FLM: Fresh liver mass, (G) RHB: Ratio of heart mass to body mass, (H) RLB: Ratio of liver mass to body mass, (I) RHS: Ratio of hindlimb length to SUL. Data were analysed using one-way ANOVA followed by the LSD post hoc test. Significant differences among altitudinal groups were indicated by different alphabetic letters.

3,500 m, 3,500-4,000 m, 4,000-4,500 m or 4,500-5,000 m, to examine geographic morphometric variation and any relationships between altitude, sex and phenotypic traits. Data were analysed using SPSS v.22.0 software (SPSS Inc., USA), and visualised using GraphPad Prism v.8.0.2 software (GraphPad Inc., USA). Before analysis, all morphological data were tested and shown to display normal distribution. Group differences in BM were analysed using two-way ANOVA (sex \times altitude). Differences in SUL, HL, RR, FLM, FHM, RLB, RHB, and RHS were assessed using two-way ANCOVA (sex \times altitude), with BM as a covariate. Similarly, differences in BM for each sex were assessed using one-way ANOVA, with differences in SUL, HL, RR, FLM, FHM, RLB, RHB,

and RHS assessed using one-way ANCOVA, with BM as a covariate, followed by LSD post-hoc tests. Finally, Pearson's correlation was used to assess relationships between morphological traits and altitude.

Results

Effect of altitude on phenotypic traits in males

While we recorded no significant difference in BM ($F = 0.495$, $P > 0.05$; Fig. 1A), SUL ($F = 1.190$, $P > 0.05$; Fig. 1B) or RR ($F = 1.390$, $P > 0.05$; Fig. 1D) with altitude gradient in males, HL ($F = 3.674$, $P < 0.01$; Fig. 1C), FHM ($F = 2.477$, $P < 0.05$; Fig. 1E), FLM ($F = 5.820$, $P < 0.001$; Fig. 1F), RHB ($F = 3.041$, $P < 0.05$; Fig. 1G), RLB ($F = 6.316$, $P < 0.001$; Fig. 1H) and RHS ($F = 3.894$,

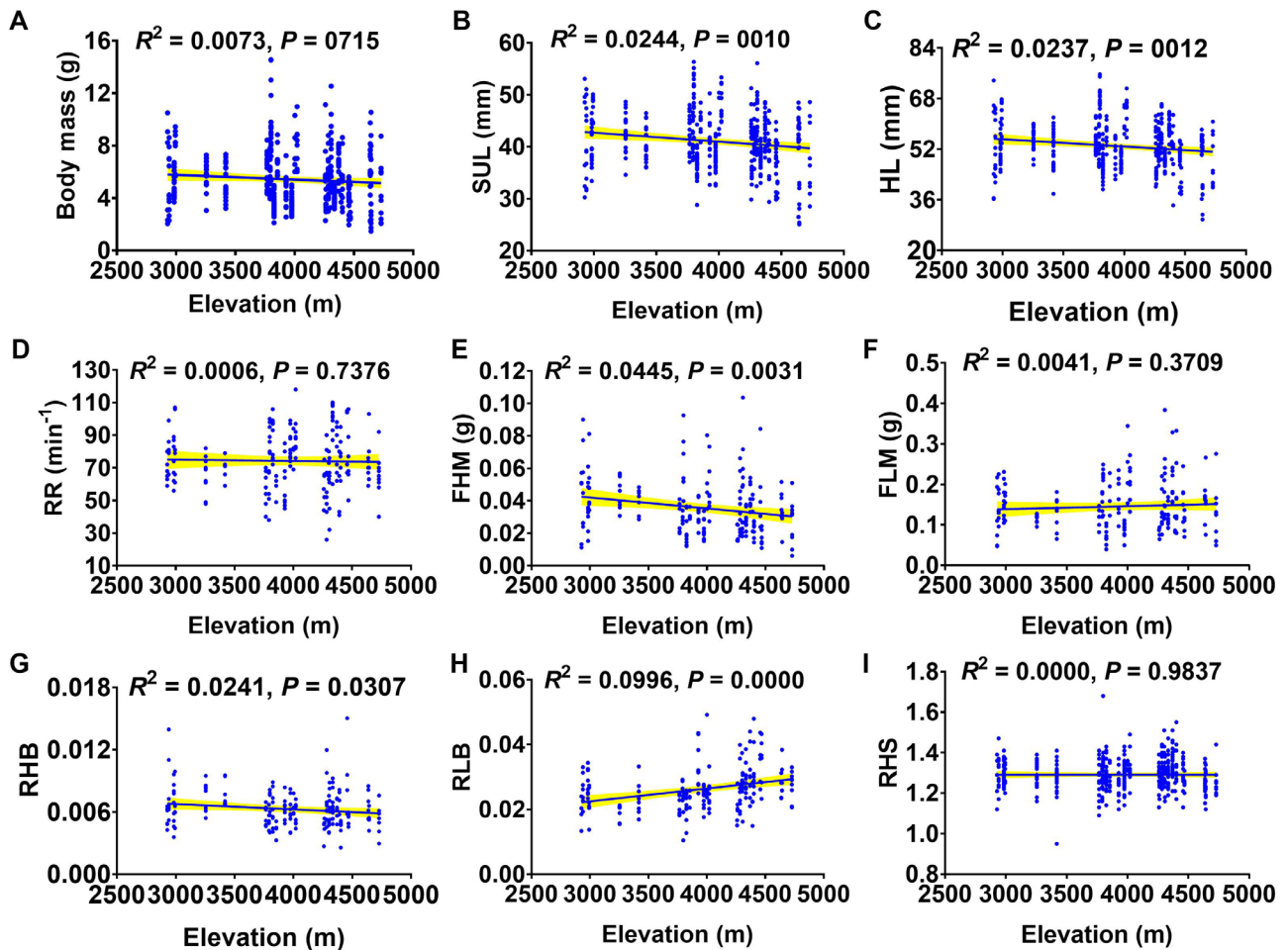


Fig. 3. Linear correlation between morphological trait and altitude in male *Nanorana parkeri*. (A) BM: Body mass, (B) SUL: Snout-urostyle length, (C) HL: Hindlimb length, (D) RR: Respiratory rate, (E) FHM: Fresh heart mass, (F) FLM: Fresh liver mass, (G) RHB: Ratio of heart mass to body mass, (H) RLB: Ratio of liver mass to body mass, (I) RHS: Ratio of hindlimb length to SUL. Data were analysed using Pearson correlation.

$P < 0.01$; Fig. 1I) all showed significant changes with increasing altitude.

Effect of altitude on phenotypic traits in females

In females, BM ($F = 3.901$, $P < 0.01$; Fig. 2A), HL ($F = 3.788$, $P < 0.01$; Fig. 2C), FHM ($F = 3.454$, $P < 0.05$; Fig. 2E), FLM ($F = 5.205$, $P < 0.001$; Fig. 2F) and RLB ($F = 3.946$, $P < 0.01$; Fig. 2H) all showed a significant increase with altitude, while there was no change in SUL ($F = 1.022$, $P > 0.05$; Fig. 2B), RR ($F = 0.937$, $P > 0.05$; Fig. 2D), RHB ($F = 2.358$, $P > 0.05$; Fig. 2G) or RHS ($F = 2.172$, $P > 0.05$; Fig. 2I).

Correlation between phenotypic traits and altitude in males

We found no relationship between BM ($R^2 = 0.0073$, $P > 0.05$; Fig. 3A), RR ($R^2 = 0.0006$, $P > 0.05$; Fig. 3D), FLM ($R^2 = 0.0041$, $P > 0.05$; Fig. 3F) or RHS ($R^2 = 0.0000$, $P > 0.05$; Fig. 3I) and altitude in males; however, SUL ($R^2 = 0.0244$, $P < 0.001$; Fig. 3B), HL ($R^2 = 0.0237$, $P < 0.001$; Fig. 3C), FHM ($R^2 = 0.0445$, $P < 0.01$; Fig. 3E), RHB ($R^2 = 0.0241$, $P < 0.05$; Fig. 3G) and RLB

($R^2 = 0.0996$, $P < 0.001$; Fig. 3H) all showed significant negative correlations with altitude.

Correlation between phenotypic traits and altitude in females

In females, BM ($R^2 = 0.0791$, $P < 0.001$; Fig. 4A), SUL ($R^2 = 0.1164$, $P < 0.001$; Fig. 4B), HL ($R^2 = 0.1510$, $P < 0.001$; Fig. 4C), FHM ($R^2 = 0.1346$, $P < 0.001$; Fig. 4E) and RHB ($R^2 = 0.0895$, $P < 0.01$; Fig. 4G) all showed significant negative correlations with altitude, with no significant relationships between altitude and RR ($R^2 = 0.0118$, $P > 0.05$; Fig. 4D), FLM ($R^2 = 0.0157$, $P > 0.05$; Fig. 4F), RLB ($R^2 = 0.0019$, $P > 0.05$; Fig. 4H) and RHS ($R^2 = 0.0017$, $P > 0.05$; Fig. 4I).

Discussion

Ectotherms in cold areas tend to grow slower and maintain larger body size, a trend known as the “temperature-size rule”, the effect differing from that resulting from limited resources (Atkinson 1994, Atkinson & Sibly 1997, Angilletta & Dunham 2003,

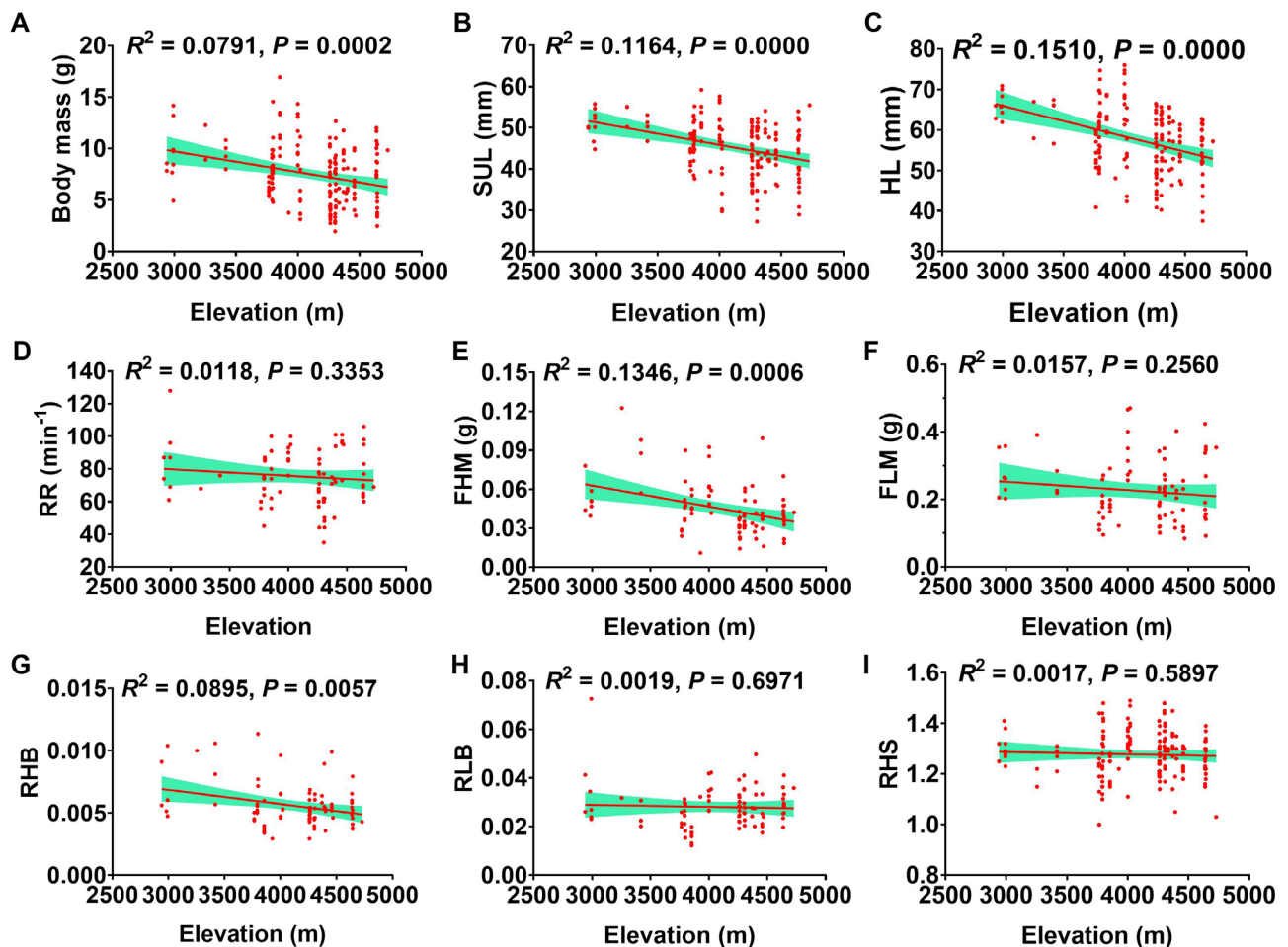


Fig. 4. Linear correlation between morphological trait and altitude in female *Nanorana parkeri*. (A) BM: Body mass, (B) SUL: Snout-urostyle length, (C) HL: Hindlimb length, (D) RR: Respiratory rate, (E) FHM: Fresh heart mass, (F) FLM: Fresh liver mass, (G) RHB: Ratio of heart mass to body mass, (H) RLB: Ratio of liver mass to body mass, (I) RHS: Ratio of hindlimb length to SUL. Data were analysed using Pearson correlation.

Angilletta et al. 2004, Kozłowski et al. 2004). At higher altitudes, *N. parkeri* have a smaller body size than those at lower altitudes, presumably due to the frog having to endure a long winter dormancy at very low temperatures on the Tibetan plateau (Niu et al. 2023). The limited energy intake allocated to maintenance, growth, hibernation and reproduction results in a slower growth rate and a smaller body size, especially in females (Ma et al. 2009, Zhang et al. 2012). The “heat balance hypothesis” proposed by Olalla-Tarraga & Rodriguez (2007) suggests that, for ectotherms, larger body size has the potential advantage of heat conservation in cold environments due to a reduced surface-to-volume ratio. On the other hand, smaller body sizes could potentially result in a net heat gain due to their greater surface-to-volume ratio and thus would be favoured in cold climates (Olalla-Tarraga & Rodriguez 2007). Our results confirm this, with *N. parkeri* at higher altitudes having smaller body sizes, enabling them to rapidly warm their bodies during their limited activity periods. In comparison, *Nanorana* sp. distributed at lower altitudes, such as

Nanorana phrynoides, tend to have a larger body size. Therefore, reducing body size in response to altitude is likely critical in allowing frogs to manage their energy reserves effectively.

Our study found that phenotypic traits in *N. parkeri*, such as morphometrics (BM, SUL, HL) and organs size (FLM, relative liver size), exhibited significant variations with increased altitude, with BM decreasing at higher altitudes in females but not in males. Moreover, while FLM decreased with altitude in both sexes, it decreased less in males than females. On the other hand, RLB increased significantly with altitude in both sexes but increased steeper in males than females. While SUL, HL and FHM showed a significant decrease with altitude in both sexes, BM and RHB showed a significant decrease in females, but not males, while RLB showed a significant decrease with altitude in males but not in females. These variations suggest that environmental selection pressures on *N. parkeri* differ between sexes and in different geographical populations, promoting the



evolution of different life strategies in geographically separated populations. High-altitude *N. parkeri* populations, for example, tend to display more robust haematological parameters, less oxidative damage and stronger antioxidant defences than low-altitude populations (Niu et al. 2022), while different adaptive strategies between sexes tend to make females more sensitive to environmental change (Zhang et al. 2012).

We observed that while the respiratory rate at different sampling localities remained consistent, body size in both sexes tended to decrease with altitude. This observation suggests that frogs at higher altitudes have a relatively larger surface area, which may make skin respiration more effective. Many ectotherms suppress their metabolic levels and reduce their energy demands in harsher environments, such as low ambient air temperatures or low oxygen partial pressure, to extend their survival (Bernardo 1994, Klaassen 1995, Hammond & Diamond 1997). In our study, FHM differed significantly between altitudinal groups, suggesting that heart size may be linked with oxygen supply at different elevations, a finding consistent with those of Zhong et al. (2017) regarding the Hong Kong whipping frog *Polypedates megacephalous*, but not those for Andrew's toad *Bufo andrewsi* (Zhao et al. 2019). The harsher environment at higher altitudes tends to restrict energy accumulation (Ma & Lu 2009); thus, the liver, as a nutritional organ linked with energy metabolism and the primary energy-storage organ in frogs (Zhong et al. 2017), tends to increase in size at higher altitudes, at least in males, allowing the frog to store more energy (Zhao et al. 2019). In our species, however, both FLM and relative liver size decreased in higher altitude populations, in line with a decrease in BM; however, this may increase its heat transfer activity and efficiency. Unfortunately, we did not measure this during the present study, but it will be the focus of future research. Overall, variations in the size of these organs differed in males and females, suggesting differing resource allocation strategies between the sexes, possibly as female *N. parkeri* are more sensitive to harsher conditions (Zhang et al. 2012). Finally, our results provide evidence at the organ level supporting the condition-dependent hypothesis (Bonduriansky 2007, Starostova et al. 2010, Zhang et al. 2012).

Allen's rule is a well-known principle stating that the appendages (limbs) of endotherms tend to increase in size with altitude or decrease in colder regions, and describes how this may be advantageous for heat conservation (Allen 1877). Indeed, anurans living in

colder, high-altitude habitats tend to have smaller appendages, which could reduce heat dissipation and prove advantageous for heat conservation when activity time or resources are limited (Mai et al. 2019). In our work, appendage length in females decreased sharply with increasing altitude, most likely helping the females to conserve energy and allocate it to other life processes through reduced heat loss. In male anurans, HL length plays a vital role in mate competition and traits associated with sexual selection (Mi 2013). This observation was reflected in the lower decrease in male HL than females with increasing altitude, confirming the adaptive pressure of sexual selection in males. In amphibians, egg production is an energy- and resource-consuming process. Females typically face greater survival pressure and reproductive costs; thus, at higher altitudes, females tend to reallocate limited energy resources and store more energy for reproduction (Ma & Lu 2009).

Life history theory is based on the idea that natural selection and trade-offs in resource allocation to different biological processes, such as maintenance, growth and reproduction, can jointly maintain phenotypic variations among geographical populations (Kozłowski et al. 2004, Dammhahn et al. 2018). Consequently, populations living in resource-limited environments should have lower metabolic rates and a slower pace of life than those occupying more productive environments (Dammhahn et al. 2018). Our study recorded different body compositions between lower and higher altitude populations and between sexes, suggesting a different pace-of-life between geographical populations and sexes. Such differences have contributed to differences in tolerance to harsh environmental conditions and different life strategies between sexes (Ma & Lu 2009, Zhang et al. 2012). For *N. parkeri*, males need to find and compete for the larger-sized females during the breeding period. Under the same conditions, therefore, (i.e. lower temperatures at higher altitudes), males will tend to require more energy to mate successfully than females. This variation is considered adaptive as allocating resources to the growth and maintenance of specific body organs entails trade-offs with other aspects of survival and reproduction (Perrin 1992). Overall, our results are consistent with studies on body size variation in this species, with female body size being more strongly affected by environmental conditions than that of males and showing a steeper decrease in BM with increasing altitude than males. Here, we suggest that geographical variation in body composition may be an essential adaptation to local environments (Corp et al. 1997, Zhong et al. 2017,



Mai et al. 2019, Zhao et al. 2019), with the size of nutritional and circulatory organs being a significant determinant of pace-of-life, daily energy expenditure and breeding success (Daan et al. 1990, Hammond & Diamond 1997).

In the present study, phenotypic traits in *N. parkeri* showed significant altitudinal variation, an adaptive mechanism that may be widespread in wild anuran populations. We also found differences in organ size between sexes at different altitudes, potentially resulting from differing environmental sensitivity between the sexes. However, while energy demand probably explains the altitudinal changes in male liver size, reduced oxygen supply did not explain changes in heart size in this frog. Thus, geographical variations in organ size in response to different ecological conditions may be an important factor underlying observed geographical variations in body size, breeding strategy and life history.

Acknowledgements

This work was supported by the National Natural Scientific Foundation of China (No. 32160254), Yunnan Ten

Thousand Talents Plan Young and Elite Talents Project (YNWR-QNRC-2019-047), and Yunnan Provincial Middle-Young Academic and Technical Leader Candidate (2019HB013).

Author Contributions

D. Hou and T. Jia contributed equally to this work. D. Hou and T. Jia were involved in interpreting data and writing the manuscript. D. Hou and Y. Ren were involved in data analysis. P. Liu was responsible for experimental design. D. Hou and W. Zhu were responsible for experimental design and manuscript revision. All authors read and approved the final version of the manuscript.

Data Availability Statement

The raw data supporting this study's findings are available in the FigShare Digital Repository: <https://doi.org/10.6084/m9.figshare.22281301.v1>.



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