

Altitudinal variation in limb size across seven populations of *Rana kukunoris* (Anura: Ranidae) fails to obey Allen's rule

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Abstract

Altitudinal variation in limb size across seven populations of *Rana kukunoris* (Anura: Ranidae) fails to obey Allen's rule. In terms of body size patterns, most research has focused on studying intra- and interspecific variation following the “Bergmannian” patterns. Allen’s rule, closely connected to these patterns, has historically been given comparatively less attention. In this study, our aim was to assess the validity of Allen’s rule, which suggests that the relative size of limb length should decrease as altitude increases. Here, we investigated the geographical variation in limb length among populations of the Plateau Brown Frog, *Rana kukunoris*, across seven distinct altitudes on the eastern Tibetan plateau. Our findings demonstrated a significant positive correlation between forelimb length of males relative to body size and altitude, contradicting the pattern predicted by Allen’s rule. In our study system, we observed a negative correlation between elevation and temperature seasonality as well as water deficit, but a positive correlation with annual total precipitation, speculating that alternative selective forces may play a role in driving the increase in extremity length at high elevations. Long forelimbs only appear in high-altitude environments, not in aggregations with a high operational sex ratio (OSR), suggesting that individuals in stressful environments (e.g., high altitude or latitude) would drive the differentiation in relative extremity length.

Keywords: Body proportion, Environmental factor, Geographical variation, Operational sex ratio, Plateau Brown Frog.

Resumo

Variação altitudinal no tamanho dos membros em sete populações de *Rana kukunoris* (Anura: Ranidae) não obedece à regra de Allen. Em termos de padrões de tamanho do corpo, a maior parte da investigação tem-se centrado no estudo da variação intra e interespecífica segundo os padrões “Bergmannianos”. A regra de Allen, intimamente ligada a esses padrões, tem merecido comparativamente menos atenção. Neste estudo, nosso objetivo foi avaliar a validade da regra de Allen, que sugere que o tamanho relativo do comprimento dos membros deve diminuir à medida que a altitude aumenta. Aqui, investigamos a variação geográfica do comprimento dos membros entre

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populações da rã-castanha-do-planalto, *Rana kukunoris*, em sete altitudes distintas no planalto tibetano oriental. Nossos resultados demonstram uma correlação positiva significativa entre o comprimento dos membros anteriores dos machos relativamente ao tamanho do corpo e à altitude, contradizendo o padrão previsto pela regra de Allen. Em nosso sistema de estudo, observamos uma correlação negativa entre a altitude e a sazonalidade da temperatura, bem como o déficit hídrico, mas uma correlação positiva com a precipitação anual total, sugerindo que forças seletivas alternativas podem desempenhar um papel na condução do aumento do comprimento das extremidades em altitudes elevadas. Os membros anteriores longos só aparecem em ambientes de grande altitude e não em agregações com uma elevada razão sexual operacional, sugerindo que ambientes estressantes (por exemplo, grande altitude ou latitude) levariam à diferenciação no comprimento relativo das extremidades.

Palavras-chave: Fator ambiental, Proporção corporal, Rã-castanha-do-planalto, Razão sexual operacional, Variação geográfica.

Introduction

Body size and body size proportions (e.g., extremity length) often exhibit ecogeographical patterns by adaptation to climate (Ballinger and Nachman 2022), such as those described by Bergmann's and Allen's rules (Bergmann 1847, Allen 1877). Bergmann's rule (1847) postulates that organisms inhabiting colder climates generally exhibit a larger body size compared to those in warmer climates. Allen's rule (1877) states that endotherms living in colder environments tend to have shorter appendages (e.g., ears, snout, tail, limbs) than those living in warmer environments (Tabh and Nord 2023). Both rules are supported by empirical studies (Ashton *et al.* 2000, Alhajeri *et al.* 2020), which show that endothermic animals with equal volume can possess varying surface areas, which can either facilitate or hinder their temperature regulation (Jin and Liao 2015). The gradient in body proportions arises from the allometric growth of prominent body parts across different geographic regions (Bidau and Marti 2008, Jin and Liao 2015, Hinckley *et al.* 2022). This phenomenon typically gives rise to latitudinal and/or altitudinal trends in body proportions, leading to the relative shortening of protruding body parts at higher latitudes and altitudes (Hinckley *et al.* 2022).

Ectotherms follow Bergmann's and Allen's rules because they are even more dependent on external temperature than endotherms (Volynchik 2014). Subsequent studies have shown that ectotherms, either vertebrate or invertebrate, may show geographic body size patterns that sometimes align with these rules' predictions (Ray 1960, Blanckenhorn and Demont 2004, Bidau and Marti 2007a,b). Especially regarding Bergmann's rule, which has primarily focused on the analysis of intra- and interspecific patterns conforming to the Bergmannian trend, it is noteworthy that Allen's rule has traditionally received relatively less attention (Bidau and Marti 2008).

Allen's rule has been proposed to be valid in ectotherms (Ray 1960). For example, Allen's rule has been tested in a limited number of ectothermic taxa, including arthropods (Bidau and Marti 2008), amphibians (Alho *et al.* 2011), and lizards (Langkilde 2009, Jin and Liao 2015). In ectotherms, the thermoregulatory adaptation hypothesis is less clear than in endotherms. Ectotherms primarily elevate their body temperature through exposure to external sources of heat and minimize heat absorption with a relatively smaller body surface area (Alho *et al.* 2011). This characteristic can be advantageous in thermally variable environments, allowing ectotherms to avoid overheating in hot

microhabitats while conserving thermal energy in colder ones (Angilletta 2009).

Male-male competition is common in some amphibian species (Black and Brunson 1971, Davies and Halliday 1979, Lamb 1984, Reading and Clarke 1983, Hoglund 1989, Luo *et al.* 2016, Deng *et al.* 2020, de Sa *et al.* 2020). Male competition for females is expected when the operational sex ratio (OSR, the ratio of sexually competing males to fertilizable females) is male-biased (Emlen 1976, Kvarnemo and Ahnesjo 1996). As the OSR becomes increasingly biased in breeding aggregations, competitors are expected to exhibit escalating levels of aggressiveness in their efforts to defend mates (Weir *et al.* 2011). In a highly male-biased OSR, amplexant males with larger limbs form more secure pairings and are less likely to be dislodged. As a result, the size of the forelimb and hind limb of male toads may affect pairing competition (Lee 2001, Lee and Corrales 2002). Larger limbs might be expected in a highly male-biased OSR, indicative of strong competition. Relatively longer limbs in amphibians can be related to the locomotor capacity, and this, in turn, may influence the ability to avoid predators (Tejedo *et al.* 2000), help maintain communication between populations (Trochet *et al.* 2019), enhance spermatophore transfer during courtship (Morrison and Hero 2003), or aid in dispersal ability at high elevations (Phillips *et al.* 2006).

In this study, we investigated variation in limb characteristics of both female and male Plateau Brown Frogs, *Rana kukunoris* Nikolskii, 1918, along a broad elevational gradient (2000–4400 m a.s.l.) (Fei *et al.* 2012). These frogs are consequently exposed to a wide range of environmental factors. The main objectives of this work were to: (1) investigate whether the species adheres to the prediction put forth by Allen's rule, which suggests that populations at higher elevations possess relatively shorter limbs; and (2) verify whether a correlation exists between limb length and the population-specific OSR.

Materials and Methods

Study Species

Rana kukunoris is a frog species endemic to the eastern Tibetan Plateau and exhibits a broad distribution at elevated elevations (ranging from 2000 to 4400 m a.s.l.) (Fei *et al.* 2012). Similar to many other amphibian species, *R. kukunoris* exhibits female-biased size dimorphism (Yu *et al.* 2022a). Previous analysis of several populations of *R. kukunoris* found a negative relationship between body size and altitude (Yu *et al.* 2022b), which follows Bergmann's rule. This species is a typical explosive breeder (Wells 2007) and experiences a brief breeding period lasting from 9 to 21 days (Yu *et al.* 2018). Due to the colder temperatures at higher altitudes, the breeding time of high-altitude brown frogs is delayed, providing us with additional time to investigate the correlation between altitude and the development of limbs in sexually mature individuals.

Study Sites and Sample Collection

We collected a total of 417 *R. kukunoris* (157 females and 260 males) from seven populations (ranging from 2297 to 3441 m in altitude; breeding populations below 2500 m and above 3500 m are uncommon) between 2011 and 2013 during reproductive periods in the eastern Tibetan Plateau of southwest China, which includes areas such as eastern Qinghai and southern Gansu (101°34'–102°69' E, 34°29'–36°68' N; Figure 1, Table 1). Annual mean temperature (AMT), annual total precipitation (ATP), temperature seasonality (TS), and precipitation seasonality (PS) were extracted from WorldClim at a resolution of $0.167^\circ \times 0.167^\circ$ grid cells (Hijmans *et al.* 2005). AET and PET were extracted at a grid cell resolution of $0.5^\circ \times 0.5^\circ$, following the methodology of Willmott and Matsuura (2001). In these locations, we observed a positive correlation between altitude and ATP by calculating Pearson's

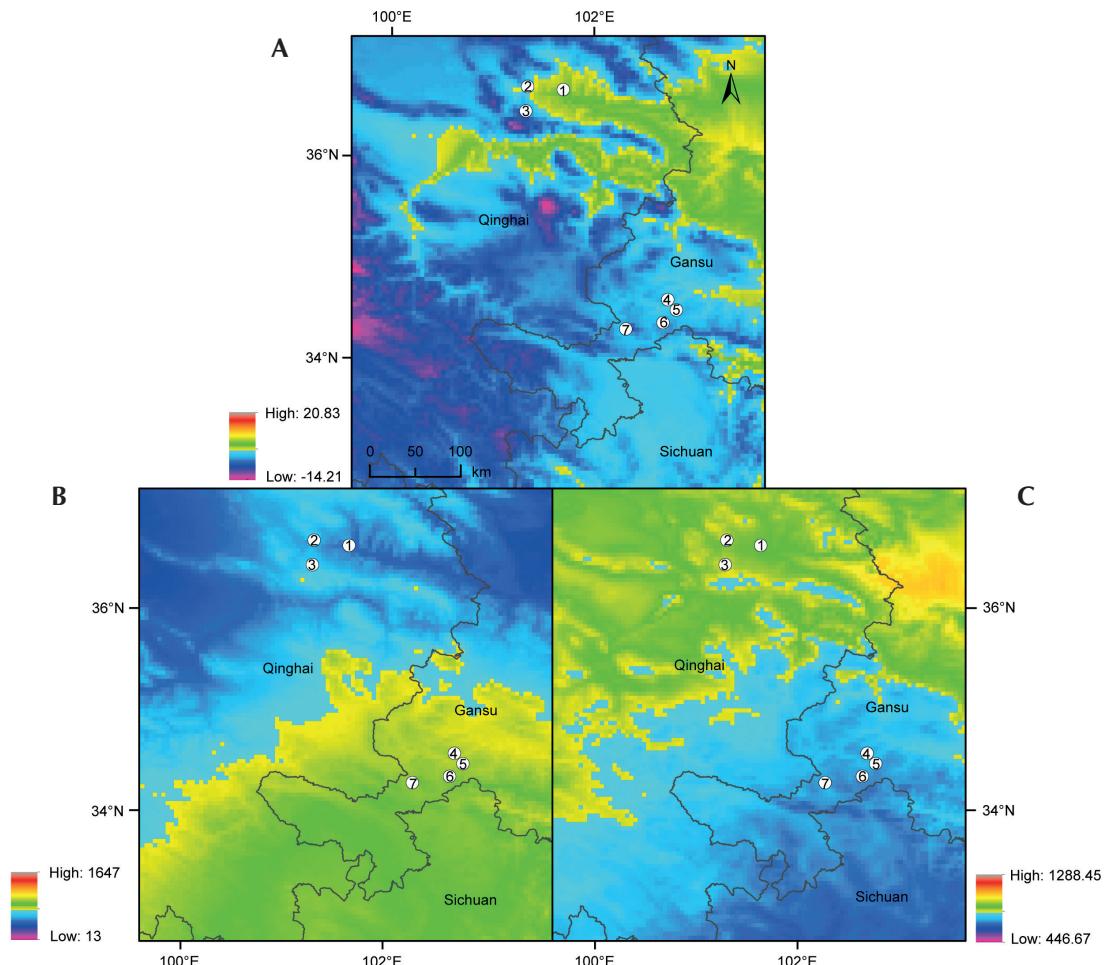


Figure 1. Topographic map showing the location of the seven study populations of *Rana kukunoris* on the eastern Tibetan plateau. Climatic layers are (A) annual mean temperature, (B) annual total precipitation, and (C) temperature seasonality.

correlation coefficients, while there was a negative correlation between TS and water deficit (WD, PET minus AET) (Table 2). For all sites, frogs were captured randomly and by hand with gloves every other day during their breeding seasons, particularly when they were in amplexus or actively seeking mates. Usually, sampling at each site was carried out over a period of one to three days. We verified the maturity of all individuals by directly observing the secondary

sexual characteristics, such as the presence of nuptial pads on the first finger in males or an enlarged abdomen for females. Furthermore, owing to the reduced water temperature experienced during the breeding season, most sexually mature individuals congregate at the breeding ponds.

Snout-vent length (SVL), forelimb length (FL) measured from the axilla to the tip of the longest toe on the left forelimb (Yu and Lu

Table 1. Study site details, including altitude, latitude, environmental predictors, morphological traits, and sample sizes for seven high-altitude populations of *Rana kukunoris* in the Tibet Plateau. SVL: Snout-vent length, AMT: Annual mean temperature, TS: Temperature seasonality, ATP: Annual total precipitation, PS: Precipitation seasonality, PET: Potential evapotranspiration, AET: Actual evapotranspiration, WD: Water deficit.

Sites	Altitude (m)	Longitude (degrees)	Latitude (degrees)	OSR	Sex	SVL (mm)	Forelimb length (mm)	Hind leg length (mm)	AMT	TS	ATP	PS	PET	AET	WD
1. Dabaoz'i'cun	2297	101.65	36.65	1.55	F	61.75 ± 6.84(8)	30.81 ± 4.46(8)	77.05 ± 7.07(8)	5.3	3347	399	92	45.07	30.93	14.138
2. Shiyaz'huang	2594	101.34	36.68	1.93	F	62.56 ± 7.23(20)	28.10 ± 3.16(20)	73.08 ± 6.40(20)	3.5	8374	419	95	34.45	30.37	4.079
3. Damoshi'cun	2789	101.44	36.49	2.00	F	61.19 ± 6.48(70)	28.41 ± 4.42(70)	73.29 ± 7.45(70)	0.2	7965	440	94	31.83	28.27	3.559
4. Zechazhan	3049	102.69	34.49	1.97	F	66.05 ± 4.49(20)	32.55 ± 3.07(20)	78.40 ± 5.78(20)	1.5	7198	614	84	29.71	29.7	0.003
5. Shibadao'wan	3060	102.69	34.47	1.67	F	61.59 ± 6.27(11)	30.45 ± 3.78(11)	75.55 ± 6.71(11)	1.4	7178	620	84	29.71	29.70	0.003
6. Shilin' zhan	3188	102.68	34.37	2.20	F	58.66 ± 5.40(28)	29.36 ± 4.73(28)	73.43 ± 4.35(28)	1.0	7129	631	85	29.71	29.7	0.003
7. Guoguo' ri	3441	102.31	34.29	1.88	M	53.33 ± 2.93(10)	28.85 ± 2.73(10)	67.90 ± 4.91(10)	0.8	7125	642	87	31.95	31.94	0.005

Table 2. Correlation coefficients between each environmental variable. AMT: Annual mean temperature, TS: Temperature seasonality, ATP: Annual total precipitation, PS: Precipitation seasonality, PET: Potential evapotranspiration, AET: Actual evapotranspiration, WD: Water deficit. ** $p < 0.001$, * $p < 0.05$.

Parameters	Altitude	AMT	TS	ATP	PS	PET	AET	WD
Altitude		-0.806*	-0.919**	0.923**	-0.713	-0.796*	0.142	-0.878**
AMT	-0.806*		0.704	-0.634	0.417	0.879**	0.407	0.845*
TS	-0.919**	0.704		-0.988**	0.923**	0.742	-0.048	0.801*
ATP	0.923**	-0.634	-0.988**		-0.925**	-0.722	0.159	-0.803*
PS	-0.713	0.417	0.923**	-0.925**		0.549	-0.094	0.606
PET	-0.796*	0.879**	0.742	-0.722	0.549		0.382	0.979**
AET	0.142	0.407	-0.048	0.159	-0.094	0.382		0.184
WD	-0.878**	0.845*	0.801*	-0.803*	0.606	0.979**	0.184	

2010), and hindlimb length (HL) measured from the posterior midline trunk to the tip of the fourth toe on the left hindlimb (Xia *et al.* 2011) were recorded for each captured frog. The measurements were carried out using a plastic ruler, with an accuracy of up to 1 millimeter. During the process, efforts were made to fully extend the frog's limbs. To minimize any potential errors, all measurements were conducted by the same person (TLY). Body mass of each frog was measured using an electronic balance to the nearest 0.01 g. Measured single or paired frogs were temporarily placed in a designated holding container within the field laboratory, such as a 15-Liter capacity barrel, to prevent duplicate sampling and the transmission of diseases. During the three-years study period, five of the six populations were surveyed only once, thereby substantially diminishing the likelihood of duplicate sampling. Upon completion of all necessary field measurements, individuals were promptly and safely returned to their original spawning grounds, ensuring their return to their natural habitat with minimal interference.

We calculated operational sex ratio (OSR) within each population. OSR was determined by

dividing the number of males by the number of females capable of reproduction (i.e., those capable of carrying eggs) present at a breeding site over a specific period, usually spanning several days (Emlen and Oring 1977).

Statistical Analyses

We used log10 transformation of the SVL, FL, and HL of each individual to achieve normality. The One-Sample Kolmogorov-Smirnov Test was used to test whether data met normal distribution. We used one-way analyses of variance (ANOVAs) and Welch's t-tests to analyze variation of SVL, FL, and HL between populations or between sexes. Linear mixed models (LMMs) were used to quantify the relationship between FL/HL and SVL. To test for altitudinal variation of FL/HL we used LMMs where FL/HL was treated as the dependent variable, SVL, and altitude as covariates, and population as a random effect.

We used LMMs where FL/HL was treated as the dependent variable, SVL, altitude, and OSR as covariates, and population as a random effect to test whether FL/HL covaried with OSR, as an indicator of competition levels. In the subsequent

analyses of reproductive traits against competition levels, altitude was not included in the simplified models because it was a non-significant fixed effect. The software IBM SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA) was used for all analyses.

Results

Mean body size, FL, and HL differed significantly among the populations for each sex (male, SVL, $F_{5,254} = 12.381, p < 0.001$; FL, $F_{5,150} = 7.171, p < 0.001$; HLL, $F_{5,254} = 30.310, p < 0.001$; female, SVL, $F_{5,151} = 13.251, p = 0.008$; FL, $F_{5,151} = 3.532, p = 0.005$; HLL, $F_{5,151} = 2.386, p = 0.041$). Females were significantly larger in SVL, FL, and HL than males in 4, 2, and 3 of 5 populations (all $p < 0.05$). LMMs showed that mean body size of both sexes was not significantly negatively correlated with altitude (male: $t = -1.265, df = 4.609, p = 0.266$; female: $t = -0.296, df = 5.253, p = 0.779$) when controlling for population (random effect: $Z = 1.280, p = 0.200$; $Z = 1.051, p = 0.293$).

Further, LMMs showed that FL (male: $t = 7.728, df = 153.844, p < 0.001$; female: $t = 5.052, df = 154.640, p < 0.001$) and HL (male: $t = 11.546, df = 255.107, p < 0.001$; female: $t = 9.079, df = 150.588, p < 0.001$) of both sexes was significantly positively correlated with SVL when controlling for population (MFL, random effect: $Z = 1.369, p = 0.171$; MHLL, $Z = 1.541, p = 0.123$; FFL, random effect: $Z = 0.983, p = 0.325$; FHLL, $Z = 0.646, p = 0.518$). After removing the effect of SVL (male, fixed effect: $t = 8.299, df = 142.177, p < 0.001$; female, $t = 5.075, df = 152.704, p < 0.001$) and population (male, random effect: $Z = 0.957, p = 0.339$; female, $Z = 0.795, p = 0.427$), the LMMs revealed that FL of males was significantly positively correlated with altitude ($t = 2.954, df = 5.439, p = 0.029$; Figure 2), but FL of females was not correlated with altitude ($t = 0.796, df = 4.831, p = 0.464$). When

controlling for the effect of SVL (male, fixed effect: $t = 11.568, df = 254.334, p < 0.001$; female, $t = 9.097, df = 143.322, p < 0.001$) and population (male, random effect: $Z = 1.371, p = 0.170$; female, $Z = 0.434, p = 0.664$), the LMMs revealed that HL was not correlated with altitude (male, $t = 0.729, df = 4.044, p = 0.506$; female, $t = 0.688, df = 3.953, p = 0.530$; Figure 2).

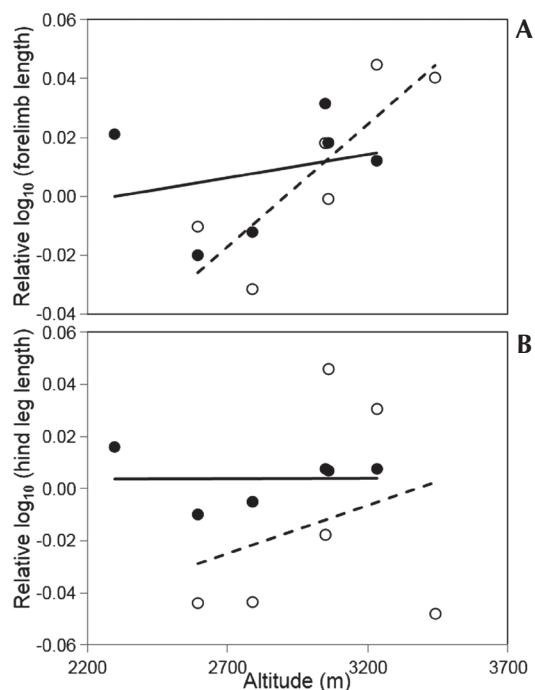


Figure 2. Relationship between altitude and (A) forelimb length and (B) hind limb length in seven populations of *Rana kukunoris*. Solid lines (filled circles): females; dashed line (open circles): males. Data points are female and male population means. Relative log₁₀ was generated from regression of log₁₀ (forelimb length) or log₁₀ (hind limb length) on log₁₀ (male body size).

The LMMs showed that FL (male: $t = 0.834, df = 6.218, p = 0.435$; female: $t = -1.640, df = 3.492, p = 0.187$) or HL (male: $t = -0.206, df = 2.934, p = 0.850$; female: $t = -1.479,$

$df = 3.695, p = 0.219$) was not correlated with the OSR when controlling for population (MFL, random effect: $Z = 0.885, p = 0.376$; FFL, $Z = 0.385, p = 0.700$; MHLL, random effect: $Z = 1.196, p = 0.232$; FHLL, $Z = 0.134, p = 0.894$) and the fixed effect of SVL (MFL, $t = 8.317, df = 146.979, p < 0.001$; FFL, $t = 5.111, df = 142.289, p < 0.001$; MHLL, $t = 11.574, df = 254.008, p < 0.001$; FHLL, $t = 9.032, df = 131.632, p < 0.001$), and altitude (MFL, $t = 2.757, df = 4.623, p = 0.043$; FFL, $t = 1.922, df = 2.828, p = 0.156$; MHLL, $t = 0.395, df = 3.012, p = 0.719$; FHLL, $t = 1.627, df = .679, p = 0.213$). In a reduced model controlling only for population (MFL, random effect: $Z = 1.231, p = 0.218$; FFL, $Z = 0.969, p = 0.333$; MHLL, random effect: $Z = 1.387, p = 0.166$; FHLL, $Z = 0.969, p = 0.333$), and SVL (MFL, $t = 7.953, df = 152.997, p < 0.001$; FFL, $t = 4.982, df = 153.886, p < 0.001$; MHLL, $t = 11.555, df = 254.680, p < 0.001$; FHLL, $t = 4.982, df = 153.886, p < 0.001$), FL or HL did not increase with the OSR (MFL, $t = 0.677, df = 5.330, p = 0.527$; FFL, $t = -0.515, df = 5.987, p = 0.625$; MHLL, $t = -0.191, df = 3.953, p = 0.858$; FHLL, $t = -0.634, df = 6.870, p = 0.546$).

Discussion

Based on Allen's rule, we would anticipate that natural selection would favor relatively shorter extremities in colder environments, leading to a negative correlation between altitude and relative limb length. However, our research outcomes obtained from the study on the wild population of Plateau Brown frogs contradicted this simple prediction, as we observed a clear linear correlation between altitude and forelimb length of males after adjusting for snout-vent length. This correlation indicates the presence of an underlying environmental pattern that produces a pattern that is the opposite of Allen's rule.

Ectotherms with varying surface area to volume ratios exhibit varying rates of heat

exchange, potentially leading to disparate selection pressures in distinct thermal environments (Jin and Liao 2015). The relative lengths of extremities, particularly limb segments, in four lacertid species—*Phoenicolacerta laevis* (Gray, 1878), *Ophisops elegans* (Ménétries, 1832), *Acanthodactylus boskianus* (Daudin, 1802), and *Mesalina guttulata* (Lichtenstein, 1823)—have been observed to generally increase in hotter environments in accordance with Allen's rule (Volynchik 2014). Amphibians, as thermoconformers, are typically dependent on moist conditions due to their permeable skin, which poses a risk of desiccation, and the fact that their larval life history is usually aquatic (Olalla-Tarraga and Rodriguez 2007, Ficetola *et al.* 2010). In this study system, we observed that only the relative forelimb length of males increased with elevation, deviating from the expected pattern according to Allen's rule. Our finding suggests that in amphibians, the primary purpose of limbs is evidently not thermoregulation (Alho *et al.* 2011).

The environmental effects encountered during the larval stage can also extend into the adult phase (Blouin and Brown 2000, Gomez-Mestre *et al.* 2010). Different climatic or biotic factors, such as increased rainfall and water abundance, reduced nutrient availability, decreased predation pressure, and alterations in food quality and abundance (Hinckley *et al.* 2022), may potentially encourage a shift toward more terrestrial foraging at higher elevations. This pattern is similar to the observed trend along altitudinal gradients in the Plateau Brown frog. We found a negative correlation between elevation and annual mean temperature, temperature seasonality, and potential evapotranspiration as well as water deficit, but a positive correlation with annual total precipitation, thus indicating that alternative selective forces may be responsible for driving the increase in extremity length at high elevations (Kitayama 1992).

The forelimb length of Plateau Brown frogs exhibited variation along the elevational gradient

after accounting for snout–vent length. As anticipated under the converse of Allen's rule, we observed a positive correlation between relative forelimb length and elevation. This result was consistent with previous studies (Jin and Liao 2015, Jaffe *et al.* 2016). Conversely, at higher elevations, having relatively longer forelimbs also could potentially provide several advantages. First, longer forelimbs could assist in effectively lifting the body, allowing for better maneuverability in steep terrains. Additionally, longer forelimbs could enlarge the field of view, enabling individuals to see further distances and detect potential threats such as predators or competitors. Moreover, elongated forelimbs might confer advantages during courtship by preventing frequent attempts by single males to displace amplexing males, ultimately increasing their breeding success. Long forelimbs only appear in high-altitude environments, not in aggregations with a high OSR, suggesting that harsh environments (high altitude or latitude) could drive the differentiation in relative extremity length.

In conclusion, our findings demonstrate a significant correlation between altitude and the relative lengths of forelimbs, which is inconsistent with Allen's rule. The varying environmental and biotic factors, such as increased rainfall and water abundance, reduced nutrient availability, decreased predation pressure, and alterations in food quality and abundance at high elevations, are likely interacting and influencing the observed increase in extremity length at high elevations.

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