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The directional testes asymmetry increases with temperature in seven plateau brown frog (*Rana kukunoris*) populations

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Abstract. Environmental stress is generally regarded as an important evolutionary force for promoting the differentiation of shape, structure and function of animal organs closely related to survival and reproduction. Geographical variation of temperature and corresponding change in intensity of male-male competition might drive inter-population differences in directional testes asymmetry (DTA). Here, we investigated inter-population variation in DTA of the brown frog (*Rana kukunoris*) at seven different altitudes on the eastern Tibetan Plateau. We found that the size of right testes increased with temperature, but not left testes. We also found that male age, body mass or body condition, and testis mass had not effect on DTA, suggesting that heavier or older *R. kukunoris* males or those with larger testes had not stronger DTA. The operational sex ratio did not affect DTA, but there was a positive correlation between DTA and temperature, suggesting that differences in the length of activity period and resources availability across locations may affect the energy budget of this frog, resulting in a gradual change in reproduction energy parallel to increasing temperature.

Keywords. Environmental factor, testes asymmetry, body condition, age, the brown frog.

INTRODUCTION

Environmental stress (e.g., resource availability, competition, or temperature) has generally been an important evolutionary force for promoting the differentiation of life-history traits (Blanckenhorn and Demont, 2004; Liao et al., 2015). For example, variation in energy acquisition under great differences in environmental conditions and environmental stress might lead to differences in the size, structure and function of animal organs and tissues among populations (Jönsson et al., 2009; Chen et al., 2011).

In most species of birds, the left testis mass is larger than the right one (e.g., Friedmann, 1927; Møller, 1994; Rising, 1996; Jamieson et al., 2007; Iddriss et al., 2018; Sara et al,. 2019). Møller (1994) hypothesized that the increase in size of the right testis is only to compensate for a reduced function of the left one, and thus, the degree of directional testes asymmetry (DTA) in testis size is a measure of male body condition. Moreover, the degree of DTA is correlated with age in that the older males in a population because they could allocate more energy to reproduction than younger individuals (Birkhead et al., 1997; Graves, 2004). Thus, there is correlation between possibilities for energy acquisition and male quality, thus the energy acquisition might have a potential impact on the degree of (DTA). In recent years, although the testis asymmetry has been proved in some anuran species (Zhou et al., 2011; Liu et al., 2011, 2012; Mi et al., 2012; Yu and Guo, 2015; Wu and Liao, 2017), there only few studies have focused on exploring geographical variation in DTA (Hettyey et al., 2005).

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Sperm competition may be attributed to drive the evolution of directional testes asymmetry. Males owning large testis mass indicate experiencing strong sperm competition when the male/female sex ratios was highly male-biased (Gage, 1994; Pitcher et al., 2005; Soulsbury, 2010; Zeng et al., 2014). For instance, compared to control treatments, males increased investment in testis mass, ejaculates or accessory glands when they lived at large population density or high male/female sex ratios (Gage, 1995; Tan et al., 2004; Ramm and Stockley, 2009). In this case, two larger testes may be more effective in increasing overall larger testes size because it might be very costly to produce directional asymmetry (Møller, 1994).

However, although relevant substantial data were collected, an understanding of the causes for geographical variation in DTA remains ambiguous and contentious. Hence, independent datasets, especially on different populations within species, that do help us to have a better understanding of the general geographical patterns of variation in male quality, age and DTA. In this study, we explore the occurrence of DTA in all study populations of the brown frog (*Rana kukunoris*), as well as the association between the degree of DTA and the body condition or age. This species is endemic to the eastern Tibetan plateau, inhabits open alpine marshes, and their habitats are located from 2200 to 4400 m in altitude. *R. kukunoris* deposits larger energy reserves in fat bodies and liver, but pre-hibernation energy stores decrease with increasing altitude (Chen et al., 2013). Further, degree of DTA was not related to altitude or body size across three populations of *R. kukunoris* (Chen et al., 2014). However, we expected a positive correlation between degree of DTA and temperature as a consequence of decreasing developmental stress (sensu Møller, 1994; Hettyey et al., 2005). Then, we expected degree of DTA increased with body condition or age. Finally, we expected degree of DTA decreased with increasing Operational sex ratio (OSR, the ratio of the sexually competing males to fertilisable females in a breeding aggregation at a given time)in response to high sperm competition level.

MATERIAL AND METHODS

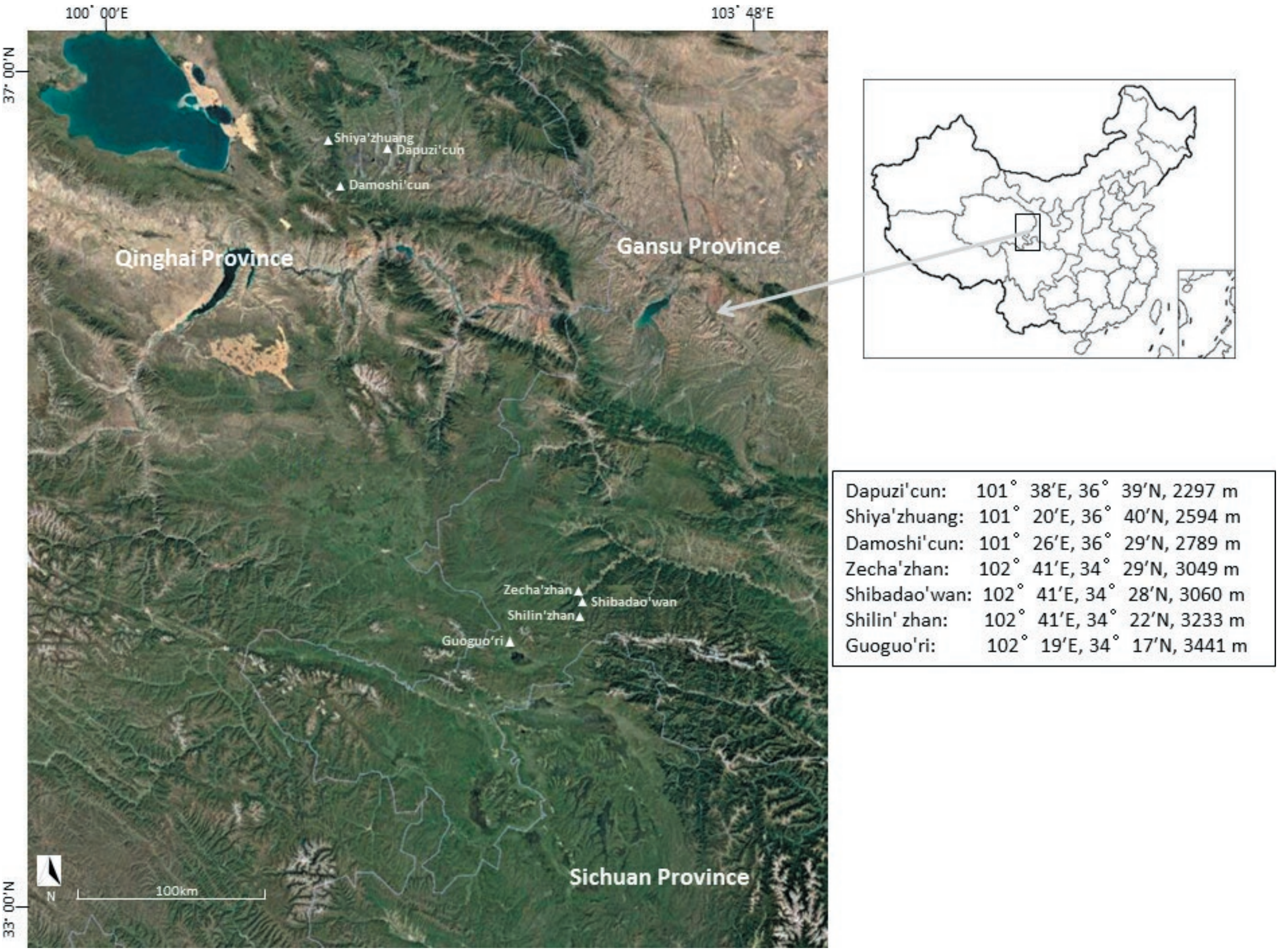
Study site and sample collection

We collected *Rana kukunoris* individuals from seven populations (elevations ranging from 2506 to 3478 m, Fig. 1) along the eastern Tibetan Plateau, China. We randomly collected 10–53 individuals by hand in the medium spawning period from late-March to mid-April in 2012 at each site. In this study, all individuals were sampled at the same time in their breeding cycle. Then, these frogs were identified as adult males if they displayed nuptial pads on the fore digits, others as females. All adult females examined were released to the original spawning sites. The population-specific OSR was calculated as the number of males to the number of fertilizable females in a breeding aggregation at a given time (3-5 days; Mai et al., 2017). In this case, OSR was used to estimate under the specific assumption that they could reflect the average number of males mating with each female. All captured males were brought to our field laboratory close to breeding sites. At room temperature, they were put into individual plastic opaque containers (diameter = 16.75 cm), filled 2 cm deep with fresh water. Then, the snout-vent length (SVL, to the nearest 0.1 mm) was measured with a vernier caliper (LXZ919160, Shenzhen luxianzi Technology Co., Ltd.), and body mass (to the nearest 0.01 g) was weighed with an electric balance (SL202N, Shanghai Minqiao Precision Scientific Instrument Co., Ltd.).

A plastic bucket was prepared with the TMS (Tricaine methane sulfonate, CAS: 886-86-2, Purity: > 97.0%, Sigma-Aldrich) at 2g/L in 5000 mL fresh water aerated for at least 48 h for dechlorination and oxygenation (Padduano et al., 2013). Then, every five frogs were put into this plastic bucket containing the 5000 mL anesthetic bath. The degree of sedation was assessed through testing of the limb retraction reflex in response to gentle pinching of a toe. Once anesthesia was achieved, adult males were euthanized by two-pithing and dissected (Liao et al., 2016). Both testes were removed and then weighed them to the nearest 0.1 mg with an electronic balance (CAV264C, OHAUS instrument (Shanghai) Co., Ltd.). Following the protocol of Hettyey et al. (2005) and Chen et al. (2014), directional testes asymmetry (DTA) was calculated with the following equation: DTA = right testis mass - left testis mass.

Age determination

We removed the longest phalange of the left hindfoot of male adults in each population and preserved in 10% aqueous solution of formaldehyde. Following the protocol of Ma et al. (2009), we produced histological sections of the frog phalanges and determined age by counting the number of lines of arrested growth (LAG) in the sections. Numerous studies have confirmed that improved method of paraffin section and Ehrlich's haematoxylin stain display seasonal growth of amphibian species (e.g., Yu and Lu, 2013; Yu et al., 2019; Yu et al., 2021).



Dapuzi'cun:	101° 38′E, 36° 39′N, 2297 m
Shiya'zhuang:	101° 20′E, 36° 40′N, 2594 m
Damoshi'cun:	101° 26′E, 36° 29′N, 2789 m
Zecha'zhan:	102° 41′E, 34° 29′N, 3049 m
Shibadao'wan:	102° 41′E, 34° 28′N, 3060 m
Shilin' zhan:	102° 41′E, 34° 22′N, 3233 m
Guoguo'ri:	102° 19′E, 34° 17′N, 3441 m

Fig. 1. Topographic map showing the location of the 7 *Rana kukunoris* study populations in the eastern Tibetan plateau.

Environmental factor collection

The annual mean temperature did not decrease significantly with elevation (Spearman's correlation: r = −0.643, P = 0.119), and latitude (r = 0.607, P = 0.148). Thus, we used annual mean temperature as environmental factor in this study. We downloaded temperature data from WorldClim (http://www.worldclim.org; Hijmans et al., 2005). WorldClim data were for the period of 1950–2000 at a resolution database of 0.167° × 0.167° grid cells.

Statistical analyses

We used one-way ANOVA to test whether the male size, age, the left or right testis mass and DTA differed among populations. The body condition was measured using residuals of body mass regressed against SVL. Then, we performed a linear mixed models (LMMs) to test variation in the left or right testis mass, where the left or right testis mass as the dependent variable, body condition, OSR, temperature and age as fixed effects, population as a random effect. To test whether degree of DTA covaried with the population-specific OSR as proxies of sperm competition levels, we also performed linear mixed models (LMMs) to test variation in degree of DTA among populations, where body condition, OSR, temperature and age as fixed effects, population as a random effect. In the subsequent analyses of reproductive traits against sperm competition levels (OSR) the temperature remained in a simplified models as a covariate, but not include body condition. Prior to analyses, we log-transformed body mass, the left or right testis mass and operational sex ratio of each population to better attain normality and enhance homogeneity of variance. SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA) was used for all analyses.

Table 1. Comparisons of SVL, age, body mass, testis mass and directional testes asymmetry of *Rana kukunoris* from seven altitudes in the east Tibet Plateau, China. Values represent mean ± SE for each measure; n = number of individuals.

Population	Altitude (m)	Latitude (degrees)	Annual mean temperature (°C)	n	OSR	SVL (mm)	Body mass (g)	Age (years)	Right testis mass (mg)	Left testis mass (mg)	Directional testes asymmetry
Dapuzi'cun	2297	36.65	5.30	17	2.43	54.70 ± 0.90	17.38 ± 0.86	3.06 ± 0.13	14.26 ± 1.08	11.51 ± 1.32	2.76 ± 0.87
Shiya'zhuang	2594	36.68	3.50	39	2.79	51.27 ± 0.58	18.35 ± 0.62	3.13 ± 0.08	14.32 ± 0.76	14.05 ± 0.76	0.27 ± 0.50
Damoshi'cun	2789	36.49	0.20	53	1.23	50.25 ± 0.58	15.39 ± 0.69	2.81 ± 0.10	10.92 ± 0.72	10.97 ± 0.73	-0.05 ± 0.54
Zecha'zhan	3049	34.49	1.50	19	1.90	54.57 ± 0.59	18.23 ± 0.79	3.32 ± 0.13	12.53 ± 1.24	14.26 ± 1.21	-1.74 ± 0.85
Shibadao'wan	3060	34.47	1.40	11	1.67	51.23 ± 0.90	14.74 ± 0.72	3.45 ± 0.16	8.79 ± 1.09	9.05 ± 0.99	-0.26 ± 0.52
Shilin' zhan	3233	34.37	1.00	24	2.20	47.40 ± 0.70	12.46 ± 0.41	2.96 ± 0.14	8.07 ± 0.64	8.42 ± 0.54	-0.35 ± 0.45
Guoguo'ri	3441	34.29	0.80	10	1.48	51.74 ± 0.98	17.56 ± 0.94	3.20 ± 0.13	12.07 ± 0.99	11.64 ± 0.79	0.43 ± 1.01
F						6.44	7.64	5.41	6.44	5.41	2.91
P						< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.01

RESULTS

Mean values of male size, age, left or right testis mass, and degree of DTA differed significantly between populations (Table 1). The LMMs showed that the right testes was not correlated with the OSR (t = -2.105, P = 0.158), but increased with temperature (t = 3.284, P = 0.042, Table 1, Fig. 2), body condition (t = 5.447, P < 0.001) and age (t = 3.235, P = 0.001) when controlling for population (random effect: Z = 0.537, P = 0.591). Similarly, the left testes was not correlated with the OSR (t = -0.262, P = 0.809) and temperature (t = 0.643, P = 0.558, Fig. 2), but increased with body condition (t = 6.361, P < 0.001) and age (t = 5.564, P < 0.001) when controlling for population (random effect: Z = 1.077, P = 0.281).

The degree of DTA was not significant correlated with age (t = -1.540, P =0.125), body mass (t = 0.788, P = 0.432) and testis mass (t = 0.107, P = 0.915), when controlling for population (random effect: Z = 1.057 P = 0.290).

The LMMs showed that the degree of DTA was not correlated with the OSR (t = -1.623, P = 0.248), body

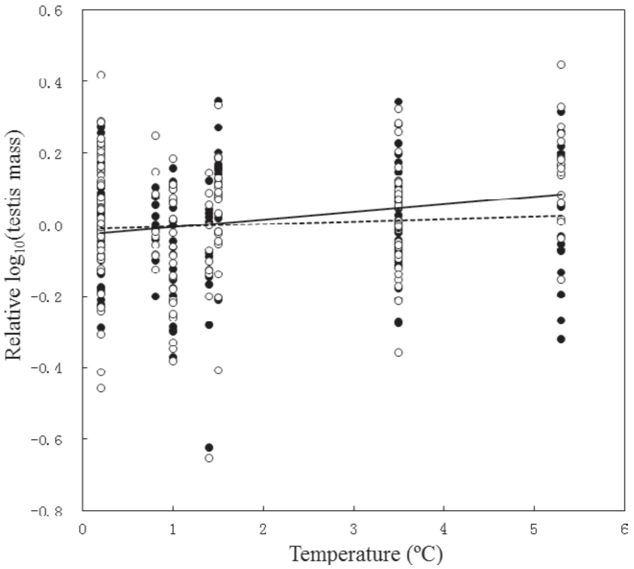


Fig. 2. Relationship between annual mean temperature and left testis mass (open circles) and right testis mass (solid circles) in 7 *Rana kukunoris* populations. Solid lines: the right testes and P < 0.05; dashed line: the left testes and P > 0.05. P values were computed using linear mixed models. Each dot represents the residual value for a given individual corrected for the effect of operational sex ratio, body condition and age.

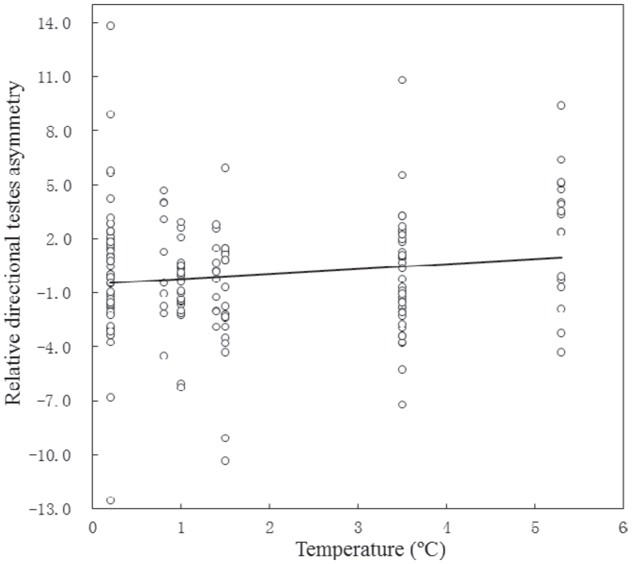


Fig. 3. Relationship between annual mean temperature and directional testes asymmetry in 7 *Rana kukunoris* populations. Each dot represents the residual value for a given individual corrected for the effect of operational sex ratio, body condition and age.

condition (t = -0.108, P = 0.914) and age (t = -1.369, P = 0.173), but increased with temperature (t = 2.899, P = 0.053; Fig. 3) when controlling for population (random effect: Z = 0.280, P = 0.780). Meanwhile, in a simplified model controlling only for population (Z = 0.253, P = 0.800), age (t = -1.377, P = 0.171) and temperature (t = 2.952, P = 0.050), DTA did not increase with the OSR (t = -1.676, P = 0.244). Furthermore, the right testis was significantly heavier than the left testis in only one of seven studied populations (5.3°C, paired test: t = 3.165, df =16, p = 0.006; other population, p = 0.055-0.928, Table 1) where the temperature was warmest among all the studied locations.

DISCUSSION

DTA is common in male animals (Møller, 1994; Simmons and Kotiaho, 2002). Specially, this phenomenon has been proved in some anurans, including *Rana temporaria* (Hettyey et al., 2005), *Rhacophorus omeimontis* (Mi et al., 2012) and *Rana nigromaculata* (Zhou et al., 2011). The degree of DTA may be used to reveal individual quality (Møller, 1994; Simmons and Kotiaho, 2002) because some studies found that there were positive correlations between DTA and body condition (e.g., Møller, 1994; Hettyey et al., 2005). However, other studies (e.g., *Rana omeimontis,* Mi et al., 2012; *Hylarana guenther*, Liu et al., 2011; *Rana kukunoris*, Chen et al., 2014) do not show this pattern. In this study, our results showed that DTA did not co-vary with male condition or body mass, suggesting individuals with good condition or heaver did not tend to have a larger degree of DTA. Thus, the degree of DTA as a good measure of male body condition remains ambiguous and contentious in male animals (Birkhead et al., 1997; Kimball et al., 1997; Kempenaers et al., 2002; Wu and Liao, 2017).

Environmental stresses may drive the evolution of testis asymmetry (Møller, 1994). Although directional asymmetry in testis size has been observed in some anurans within a population (Mi et al., 2012; Zhou et al., 2011; Yu and Guo, 2015), most studies so far have found the level of DTA was not correlated with latitude or altitude in spite of large differences in environmental conditions and environmental stress (Hettyey et al., 2005; Chen et al., 2014; Zhang et al., 2018). However, our results support this hypothesis as the degree of DTA covaried with temperature in the study populations of *Rana kukunoris*. Furthermore, the right testis was significantly heavier than the left testis in the populations with locations of warmest temperature. One possible explanation is that adaptive asymmetry might be difficult to evolve. For example, the development of testes asymmetry is adaptive, but very costly because reduction or loss of an organ on one side of the body could commonly not be compensated, leading to physiological or morphological handicaps. Males living in warm regions could allocate more energy to reproduction, in that they increase resource availability in relative longer the length of the activity seasons than those living in cold regions. This suggests that low environmental stresses (e.g., warm temperature) provide the opportunity for males to increase in size of the right testis, thus compensate for a reduced function of the left one. In addition, we found no evidence for a geographical trend in genetic stress, and this result was consistent with other anurans species (Palo et al., 2003; Hettyey et al., 2005).

Previous studies have shown that DTA covaries with age (e.g., Birkhead et al., 1997; Graves, 2004; Liu et al., 2012), suggesting that males with a larger degree of DTA have a longer life span. However, the degree of DTA was not positively correlated with age in current study. This result was consistent with Liu et al. (2011) and Zhou et al. (2011), suggesting that older males did not indicate a higher degree of DTA than younger males. An explanation for this phenomenon was males have already reached complete reproductive maturity during the first breeding year (Liu et al., 2011).

Sperm competition is also attributed to drive the evolution of ejaculate quality in a wide range of taxa, in that higher levels of sperm competition tend to result in larger testes (Møller, 1991; Møller and Briskie, 1995). Assuming that it is costly to have high the level of DTA, thus two large testes might be advantageous to increase testes size. However, we found annual mean temperature did not effect on testis mass (Yu, unpublished data). Moreover, our results also showed that degree of DTA did not decrease with the OSR, suggesting that male–male competition did not lead to an increase in levels of sperm competition.

In conclusion, we did not find evidence for the suggestion that DTA is related to male condition, age and OSR, although there is a significant directional testes asymmetry in *R. kukunoris.* We found a positive correlation between the level of DTA and temperature, suggesting that differences in the length of activity period and resources availability across locations may affect the energy budget of this frog, resulting in a gradual change in reproduction energy parallel to increasing temperature.

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The Gahai-Zecha National Nature Reserve Management Bureau approved this project (approval number GHZCRMB/03-212014), and gave permission for fieldwork. Handling and processing of frogs followed approved protocols from the Animal Scientifc Procedures Act 1988 by the State Department of China. All experiments involving the sacrifice of live animals were approved by the Animal Ethics Committee at Xinyang Normal University. The study was funded by Emergency Management Program of National Natural Science Foundation of China (Grant no. 31741019).

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