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Altitude decreases testis weight of a frog (*Rana kukunoris*) on the Tibetan plateau

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Producing sperm is energetically inexpensive, and strong competition for mating partners can lead to increased size of the testes in an effort to enhance reproductive success. On the other hand, selection on testes size can also be imposed by environmental conditions. We studied altitudinal variation and directional asymmetry in testis weight in a high-altitude frog (*Rana kukunoris*) endemic to the Tibetan plateau (2300–3500 m altitude). Testis weight decreased with increasing altitude and body size. The left testis was significantly larger than the right testis for all populations, and relative asymmetry between testes was unrelated to altitude or body size. The harsh environmental conditions at high altitudes may constrain the ability of males to allocate energy towards increased testis size. They could also be associated with altered operational sex ratios, thus reducing the strength of male-male competition.

Key words: altitude, Anura, directional asymmetry, energy allocation, *Rana kukunoris*, reproduction, testes

INTRODUCTION

Current reproductive effort can influence future reproductive success and survival (Lack, 1966). Typically, there is a negative relationship between the allocation of energy into current reproduction and future fecundity, resulting in a negative relationship between reproductive investment and survival (e.g., Hettyey et al., 2005). Constraints on resource availability often result in trade-offs in how energy is divided into growth, maintenance and reproduction (Lack, 1966). Energy allocation of females has been well-studied, because producing eggs and providing parental care are substantial investments. Much less is known about how male animals invest in reproduction, because sperm are energetically inexpensive to produce (Wells, 2007). Previous studies have detected between-population variation in male reproductive investment mediated by variation in testis size (Harcourt et al., 1981; Hettyey et al., 2005; Jamieson et al., 2007). Males generally have larger testes in populations with high male-male competition (Emerson, 1997; Byrne et al., 2002) and strong variation in female fecundity (Emerson, 1997), with testis size being further influenced by environmental conditions (e.g., duration of the reproductive season: Hettyey et al., 2005).

Testes produce sperm and secrete testosterone (Lake, 1981). Testis weight, relative to overall body size, is widely used as a measure of reproductive investment, and is under strong sexual selection (Byrne et al., 2002). Males with larger testes may enhance reproductive success by increasing sperm production (Parker, 1998), which is beneficial particularly under high male-male competition (Harcourt et al., 1981; Møller & Birkhead, 1989; Kusano et al., 1991; Gage, 1994; Hosken, 1997; Stockley et al., 1997; Byrne et al., 2002; Dziminski et al., 2009; Liao et al., 2011).

Males can increase the size of one or both of their paired testes, leading to patterns of asymmetry (Scharer & Vizoso, 2007). The testes asymmetry hypothesis (Møller, 1994) predicts that directional asymmetry in testis size should be common because one testis may grow more to compensate for a reduced function in the other testis (Møller, 1989; Birkhead et al., 1997; Briskie & Montgomerie, 2006; Scharer & Vizoso, 2007). Strong support for this hypothesis comes from birds, in which many species have a larger left testis (Friedmann, 1927; Lake, 1981; Rising, 1996; Jamieson et al., 2007). Møller (1994) predicted that the degree of directional asymmetry in testes size is a measure of health, and that only males in good body condition develop large degrees of directional asymmetry to enhance reproductive success. In anurans, male *R. temporaria* in better overall condition indeed have a larger directional asymmetry in testes size (Hettyey et al., 2005), but further studies are required to test the ubiquity of this pattern.

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Table 1. Mean and SD in body weight and testis weight for *Rana kukunoris* in three populations from the Tibetan plateau.

Parameters	Altitude (m)	*n*	Mean	SD	95% CI	
					Lower	Upper
Body mass (g)	2300	53	11.636	2.934	10.828	12.445
	2900	16	13.598	2.414	12.311	14.884
	3500	52	9.067	2.648	8.330	9.805
Body length (g)	2300	53	44.629	3.801	43.582	45.677
	2900	16	45.411	2.674	43.986	46.836
	3500	52	41.942	4.293	40.747	43.137
Left testis (g)	2300	53	0.010	0.004	0.009	0.011
	2900	16	0.009	0.003	0.007	0.011
	3500	52	0.007	0.006	0.006	0.009
Right testis (g)	2300	53	0.010	0.004	0.009	0.011
	2900	16	0.009	0.003	0.007	0.011
	3500	52	0.006	0.005	0.005	0.008
Total testes (g)	2300	53	0.020	0.008	0.018	0.022
	2900	16	0.016	0.006	0.013	0.020
	3500	52	0.012	0.007	0.010	0.014

For anurans, high altitudes represent harsh environmental conditions largely because of shortened activity seasons (Morrison & Hero, 2003). Males may show greater asymmetry in testes size when resources are limited. Many anuran populations have a male-biased sex ratio, resulting in strong competition for mates and potentially strong selection for large testis size and/or testis asymmetry (Byrne et al., 2002). In colder regions, operational sex ratios are usually more even (Wells, 1977; Hettyey et al., 2005), suggesting that some species may show a relative cline in testes size and/or asymmetry across altitudes. We studied altitudinal variation in relative testes size of *Rana kukunoris* from three populations on the eastern Tibetan Plateau (altitude: 2200–4400 m). We predicted a correlation between the level of asymmetry and altitude, because individuals from higher altitudes endure increasing developmental stress due to environmental constraints.

MATERIALS AND METHODS

Rana kukunoris inhabits open alpine marshes across the eastern Tibetan plateau, ranging in altitude from 2200 to 4400 m above sea level (Xie et al., 2000; Chen et al., 2011). In this capital breeder, females are the larger sex (Chen et al., 2011), female fecundity is positively related to body size (Lu et al., 2008; Chen et al., 2013a, b, c), and males prefer to mate with larger females (Chen & Lu, 2011). The shorter activity periods, lower food availability and unpredictable climates associated with higher altitudes may limit the opportunity for frogs to accumulate energy prior to hibernation (Chen et al., 2013b). Body size does not increase with altitude, and environmental conditions likely constrain the upper limit on body size (Chen et al., 2013a).

In 2012–2013, we collected male *R. kukunoris* early in the breeding cycle from three populations along an altitudinal gradient (Jiuzhaigou at 2300 m, 33.51°N, 102.57°E, 7.8°C; Zhuoni at 2900 m, 34.80°N, 103.23°E, 4.6°C; and Zoige at 3500 m, 33.16°N, 104.19°E, 2°C). Frogs were maintained in pond water for 24 hours to allow full hydration before being euthanised with an overdose of MS-222 (hydration status can influence animal body mass; Ladyman & Bradshaw, 2003). We measured snout-vent length (SVL; to the nearest 0.1 mm) and body mass on an electronic balance (to the nearest 0.001 g), removed both testes and weighed them. We used the sum of both testes as overall testes weight and calculated directional asymmetry as the difference between right and left testis weights (Hettyey et al., 2005).

We used general linear models (GLMs) to investigate the relationship between altitude and testis size using altitude, SVL and body mass as covariates, and testis weight (left, right and total) as the dependent variable. Interaction of all covariates were insignificant ($p>0.05$). We also used directional asymmetry as the dependent variable and altitude, SVL and body mass as covariates. To explore size differences between the right and left testes, we used paired sample *t*-tests comparing the predicted weights for the right and left testes generated from GLMs. We used partial correlation to investigate the relationship between directional asymmetry and body condition (controlling for differences among altitudes), estimated using the residual values from a linear regression using SVL as the independent variable and body mass as the dependent variable ($r^2=0.71$, $n=121$, $p<0.001$, range; -4.24–6.67; Byrne et al., 2002). Data were arc-tan transformed to improve normality of variances; all probabilities are two-tailed with a set at 0.05. Statistical tests were performed using SPSS software (v. 16.0. Chicago, SPSS Inc).

RESULTS

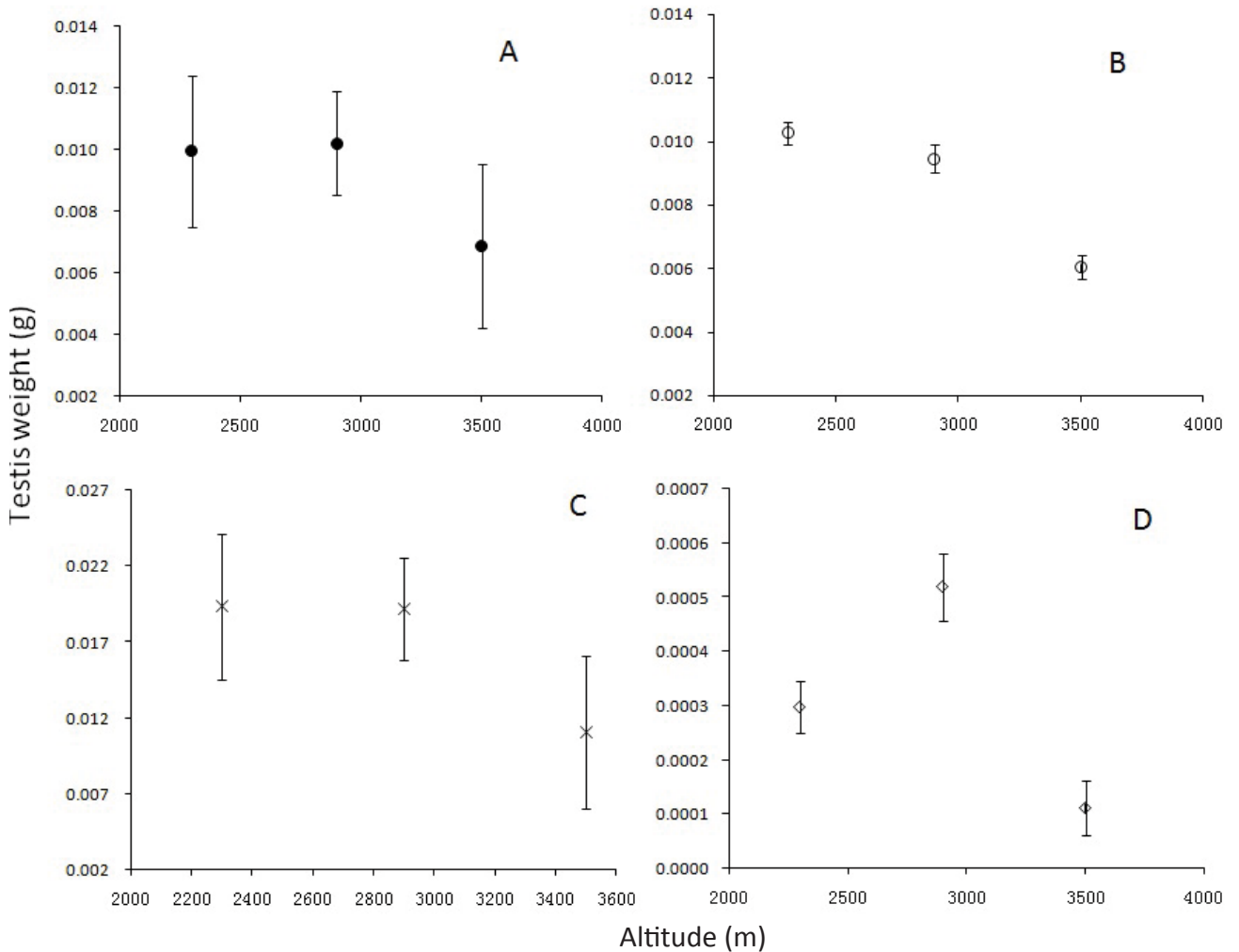We sampled a total of 53 male frogs from 2300 m, 16 from 2900 m and 52 from 3500 m. Right testis weight and

Fig. 1. Relative testes weight of *Rana kukunoris* from three populations on the Tibetan plateau, showing predicted values from General Linear Models (±SD); (A) weight of left testes, (B) weight of right testes, (C) overall weight of the two testes, (D) directional asymmetry.

overall testes weight decreased with altitude, whereas left testis weight did not differ significantly among altitudes (Tables 1, 2; Fig. 1). Larger but not heavier frogs had significantly heavier testes (right and left testes, and overall, Table 2). Directional asymmetry of the testes was not related to altitude, body length, and body mass (Table 1). The left testis was significantly heavier than the right testis for all three populations (2300 m, paired *t*=-6.68, df=52, *p*<0.001; 2900 m, *t*=7.95, df=31, *p*<0.001; 3500 m, *t*=20.46, df=51, *p*<0.001), but the degree of asymmetry was not significantly different among populations (Table 2). Directional asymmetry was not significantly correlated with body condition (*r*=0.087, *n*=134, *p*=0.315), and thus the difference in testis size appears unrelated to overall body size and/or mass.

DISCUSSION

The energetic investment allocated to reproduction reflects trade-offs between current reproductive effort and future reproductive success, as well as between current reproductive effort and survival (Lack, 1966). We found that male *R. kukunoris* at higher altitudes had smaller testes, but directional asymmetry was unrelated to altitude or body condition. Male frogs living at lower altitudes thus invest more energy in reproduction, possibly due to increased competition for mates and extended activity seasons, whereas males at high altitudes might face more relaxed competition for mates but constraints on energy acquisition from shortened activity seasons. Asymmetry in testis size and differences in testes size within and among populations are common throughout the animal kingdom (primates: Harcourt et al., 1981; frogs: Hettyey et al., 2005; birds: Jamieson et al., 2007). Breeding systems as well as environmental conditions can influence male energy allocation to reproduction (Emerson, 1997; Byrne et al., 2002; Hettyey et al., 2005), and males have larger and more asymmetrical testes when competition for mates is high and when environments are harsh (Møller, 1994).

Parker (1970) predicted that strong sperm competition can select for increased sperm production and thus increased testis weight (Harcourt et al., 1981; Schulte-Hostedde & Millar, 2004). Variation in the strength of sexual selection can help to explain the differences in relative testis size among populations (Hettyey et al., 2005). In populations with high operational sex ratios, the intensity of male-male competition should be low (Wells, 1977, 2007). Under such circumstances, smaller testes could be advantageous because individual males may

Table 2. Parameter estimates from general linear model analyses of variables for the relative testes weight of *Rana kukunoris* in three populations from the Tibetan plateau. Significant values are in *italics.* B: coefficient, CI: confidence intervals.

Parameter	B	t	p	95% CI	
				Lower	Upper
Right testis weight					
Intercept	-1.832	-4.500	*<0.001*	-2.637	-1.027
Body weight	0.004	0.197	0.844	-0.038	0.046
Body length	1.189	4.245	*<0.001*	0.635	1.742
Altitude	-0.001	-3.042	*0.003*	-0.001	-0.001
Left testis weight					
Intercept	-1.412	-3.093	*0.002*	-2.327	-0.512
Body weight	0.021	0.879	0.381	-0.026	0.068
Body length	0.905	2.866	*0.005*	0.280	1.529
Altitude	-0.001	-1.327	0.187	-0.001	0.001
Overall testis weight					
Intercept	-2.589	-4.366	*<0.001*	-3.761	-1.416
Body weight	0.039	1.277	0.204	-0.022	0.100
Body length	1.652	4.051	*<0.001*	0.845	2.459
Altitude	-0.001	-4.082	*<0.001*	-0.001	-0.001
Directional asymmetry					
Intercept	0.289	1.401	0.164	-0.119	0.697
Body weight	0.014	1.266	0.208	-0.008	0.035
Body length	-0.200	-1.411	0.161	-0.481	0.081
Altitude	0.001	0.337	0.737	-0.001	0.001

be less likely to mate successfully and therefore energy should be allocated towards growth or maintenance (Hettyey et al., 2005). High-altitude populations of *R. kukunoris* generally have female-biased operational sex ratios (Chen, unpublished data) which may have contributed to the altitudinal decline in testis weight. Although larger *R. kukunoris* females lay more eggs than smaller females, body size is similar among altitudes (Chen et al., 2013a). Males at low altitudes are likely competing more intensely for females, and robust and voluminous sperm (as should be associated with larger testes; Byrnem et al., 2002; Hettyey et al., 2005) may help to increase reproductive success.

The duration of the activity season and food availability differ substantially among our study sites (Chen et al., 2013a, b). In higher altitude populations, more energy should be allocated towards future reproduction and current survival and less energy into current reproduction (Hettyey et al., 2005). Our field data support these predictions: females produce larger eggs at high altitudes (but similar numbers of eggs), whereas males have smaller testes at high altitudes.

In most bird species, the left testis is larger than the right testis (Wright & Wright, 1944; Selander & Hauser, 1965; Rising, 1987). This pattern has also been observed in the frogs *Rana omeimontis* (Mi et al., 2012) and *R. nigromaculata* (Zhou et al., 2011), but not in *R. temporaria* (Hettyey et al., 2005). We found that the left testis of *R. kukunoris* was larger than the right testis, and

that the right testis was smaller at lower altitudes. Our findings of moderate testis asymmetry in *R. kukonoris* are consistent the directional asymmetry hypothesis (Møller, 1994). Only the left testis weight and overall testis weight were smaller at high altitudes, whereas the right testis was more reduced and similar in size among populations. Maintaining two large testes may be energetically costly but may increase performance during reproduction (Birkhead et al., 1998; Kempenaers et al., 2002; Liu et al., 2011). Individual males in good body condition may be able to develop larger degrees of directional asymmetry by increasing the size of one testis to a larger degree than males in reduced condition (Møller, 1994; Simmons & Kotiaho, 2002). Although some birds (Møller, 1994) and anurans (*R. temporaria*; Hettyey et al., 2005) support this prediction, body condition is unrelated to testis asymmetry in *R. omeimontis* and *R. kukonoris* (Mi et al., 2012; this study). Environmental stress during development, including reduced body condition due to food scarcity, can influence the sizes of reproductive organs (Kempenaers et al., 2002; Liu et al., 2011). Perhaps we did not observe this pattern because of (i) intense selection early in life (males in the poor condition die early and are unavailable to sample), (ii) males being able to compensate for reduced body condition early in life (e.g., by delaying sexual maturity) or (iii) a threshold value when body condition is too low to invest in the testis (Møller, 1994). Investigating the circumstances under which testes asymmetry of

animals reflects body condition would help understand how and when energy is invested into reproduction, and how that is influenced by environmental factors. Møller (1994) also proposed that directional asymmetry in testis weight could be a measure of developmental stress. Assuming that increased altitude results in increased stress (see also Hettyey et al., 2005), our results do not support this hypothesis. Understanding how and why males allocate energy towards reproduction, and how this differs among populations and individuals requires further experiments.

ACKNOWLEDGEMENTS

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