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Interpopulation variation in prebreeding energy reserves of plateau brown frog (*Rana kukunoris*)

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Abstract. The energy storage plays a prominent part in the life histories of many animals inhabiting cold harsh environment, thus anuran amphibians rely on larger residual energy stores from post-hibernation to cope with unfavourable weather conditions before breeding. As the habitat conditions often change in different locations, selective pressure for energy storage could also vary. However, we still have limited knowledge on whether anurans living under different environmental backgrounds would adopt suitable strategies to cope with energy demands during reproduction. Here, we examined patterns of energy storage in spring (after hibernation and before reproduction) in seven plateau brown frog (*Rana kukunoris*) populations with different climatic conditions in the eastern Tibetan Plateau. After removing the effects of age and body size, liver weights of both sexes and fat body weights in males were negatively correlated with temperature, suggesting that these reserves were critical for this anuran to survive through harsh and extended dormancy period under uncertain environmental conditions. Additionally, reproductive investment in both sexes could partly explain the increase in post-hibernation energy stores with decreasing temperature. Males had relatively heavier liver and fat body weights than females, indicating that males store more energy reserves for breeding activity compared with females. Therefore, we suggest that both the variation of environmental conditions and sexual differentiation of reproductive roles play a prominent role in shaping the energy allocation and reserve patterns in the dormancy and breeding of amphibian species.

Keywords. Energy storage, post-hibernation, plateau brown frog, Tibetan plateau.

INTRODUCTION

Acquisition and expenditure of energy are fundamental factors influencing life history traits intimately linked with fitness and population viability (Jönsson et al., 2009). Populations from environments with different constraints often evolve different patterns of energy demands and acquisition to adapt to local environmental conditions. Since the active period to breed and forage between dormancies are limited (15-30 days) for amphibians living at temperate zones or higher altitudes (> 2000m asl), energy storage strategies were expected to evolve. Therefore, energy storage plays a prominent part in the life histories of many animals inhabiting cold environments (e.g., high-altitude or high-latitude, Wells, 2007).

The organisms are usually classified as either income breeders (e.g., feeding after emergence and before breeding) or capital breeders (e.g., refrain from feeding until after breeding) based on the origin of the energy (Stearns, 1992; Jönsson, 1997; Drent and Daan, 1980). Capital breeding was widely found among ectothermic vertebrates living in cold environments that they reproduce immediately after a long hibernation (Wells, 1977; Chen et al., 2018). The energy stores before the dormancy were critical for their survival and reproductive success through the harsh winter and early spring. Generally, amphibians, such

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as burrowing amphibians or species with hibernating in water can store energy in the form of lipids, proteins and carbohydrates, but lipids should be the most important energy form because the carcass, fat bodies of abdomen and liver deposit high amounts of fat (Fitzpatrick, 1976).

Populations within species living in different environments face the environmental constraints and challenges, which may be highly variable (Gasc et al., 1997; Peng et al., 2020). In this case, high energy reserves represent a general tactic to cope with stochastic energy supply or predicted energy shortages (McNamara and Houston 1990; Jönsson, 1997). The energy demands and acquisition can determine overwintering survival rate and breeding success (Bodie and Semlitsch, 2000; Wells, 2007), compared with warm environments, anurans living in cold environments require more accumulated energy to survive the harsh and extended winter and subsequently breed in spring. Also, more residual energy stores for reproduction at post-hibernation emergence might be expected at areas with lower annual average temperature because there is a higher probability of occurrence of harsh weather conditions during early springs.

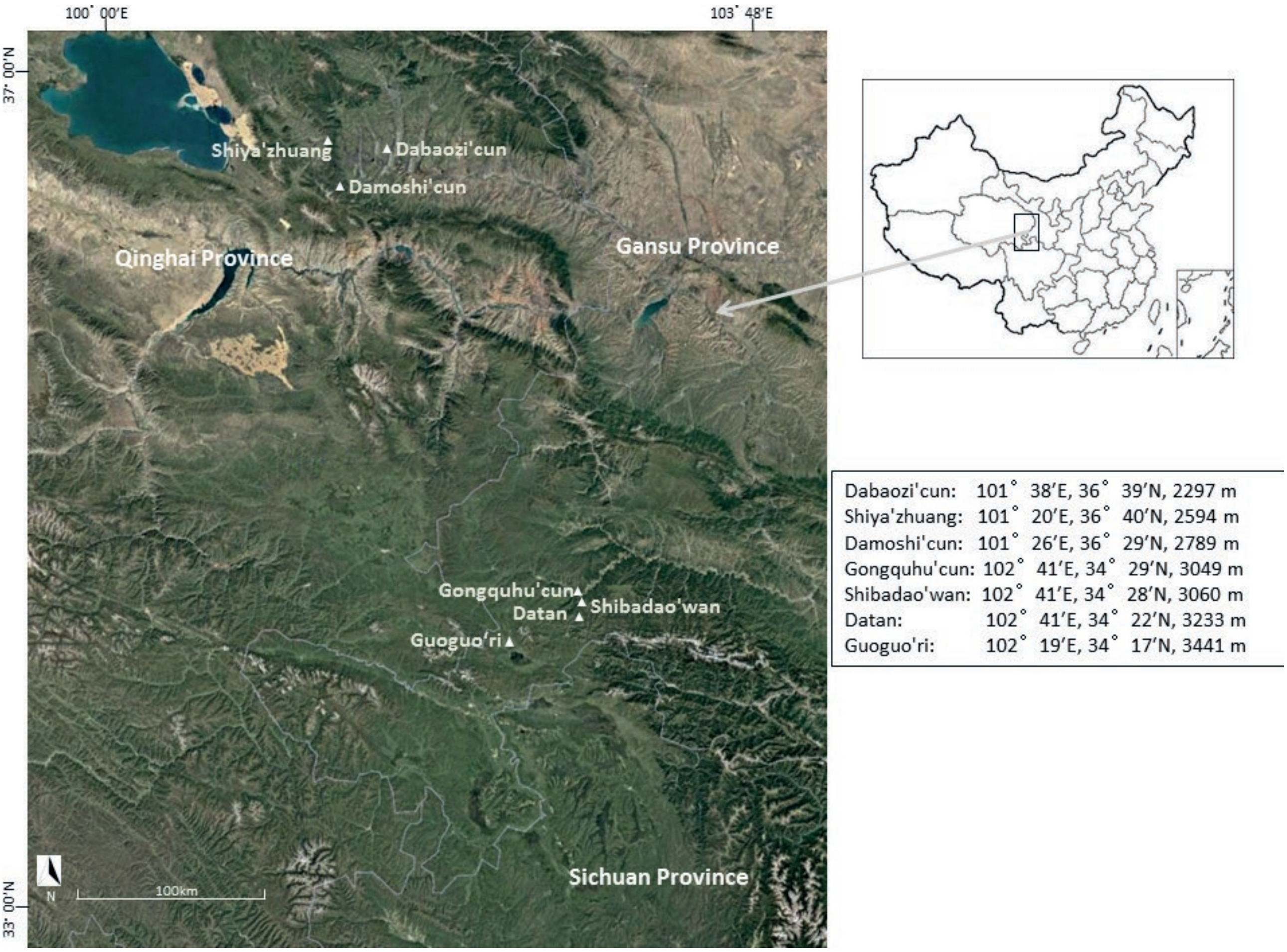
Furthermore, differences in the time of energy allocation towards reproduction between the sexes are expected to explain for sexual differences in energy reserve before breeding (Jönsson et al., 2009; Chen et al., 2011a). For example, female amphibians allocate their main energy into egg growth prior to hibernation (Lu et al., 2008; Jönsson et al., 2009), most females enter the pond unamplexed, and usually leave the pond immediately after depositing eggs. Conversely, males invest energy into breeding activities after hibernation, arriving the breeding ponds is earlier than females, usually search actively or engage in physical struggles for females until the end of the breeding season. Thus, males need larger residual energy stores and allocate more energy for demanding activities during the breeding season than females do (Wells, 1977). So far, there are limited studies exploring the patterns of energy storage based on comparisons of different populations with varied environmental conditions (see Elmberg, 1991; Elmberg and Lundberg, 1991; Chen et al., 2011a), thus we have a very poor understanding of how energy stores in the life histories varies among populations of amphibian species.

The aim of this study was to analyze the patterns of energy stores in breeding the plateau brown frog (*Rana kukunoris*) populations along an altitudinal gradient in the eastern Tibetan Plateau. This species is endemic to the eastern Tibetan plateau, inhabiting many different habitats, and their habitats are widely distributed from 2200 to 4400 m in altitude (Chen et al., 2023; Chen et al., 2023a), and thus remarkably different environmental stresses on energy stores and investments might be expected. This species is also a typical explosive breeder with shorter active period in colder regions (Wells, 2007: Chen et al., 2011b). Further, males stay in the spawning site during breeding season and do not feed, while they are forming a chorus and actively search and competing for females (Yu et al., 2013). Most of unamplexed females enter the spawning site, and usually mate with male on the same day and leave the spawning site immediately after oviposit. In this study, *R. kukunoris* enter another dormancy period after breeding because the environmental condition (e.g., cold temperature) is not suitable for individuals to feed and survive. Thus, the more energy storage before hibernation or residual energy stores for dormancy is very important for their survival and reproductive success. Here, we explored differences in organ size of liver and fat bodies in *R. kukunoris* across seven populations along an altitudinal gradient. We predicted that 1) energy stores should decrease with temperature rather than altitude since the two factors might not be strongly correlated in our studies, and 2) males should have greater energy storage before reproduction compared with females due to differences in allocation of breeding energy investment during varied periods.

MATERIALS AND METHODS

Study site and sample collection

We collected *Rana kukunoris* individuals from seven populations (elevations ranging from 2506 to 3478 m, Fig. 1; Table 1, 2) along the eastern Tibetan Plateau, China. The beginning of the breeding period for *R. kukunoris* tends to be later at higher altitudes (2297 m site: mid-March, 9 days; 2594 m site: late-March, 10 days; 2789 m site: mid-April, 15 days; 3049 m site: late-March, 11 days; 3060 m site: late-March, 10 days; 3233 m site: early-April, 11 days; 3441 m site: mid-April, 15 days, Table 1). Following the protocol of Chen et al. (2013), the frogs begin to be active when the mean daily ambient temperature is close to 6 °C. Thus, six degrees Celsius could be a threshold which is similar across study sites (unpublished data). In our study site, the length of activity period was calculated by estimating number of days with a mean daily temperature above 6 °C (Table 1). In fact, we found that *R. kukunoris* has a short dormancy period after reproduction because low temperature is not suitable for feeding activities and prey is few. Here, the length of dormancy period was calculated by estimating number of days from after breeding until mean daily temperature above 6 °C, when the length of hibernation period was calculated by estimating number of days from mean daily tempera-



Dabaozi'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
Gongquhu'cun:	102° 41'E,	34° 29'N,	3049 m
Shibadao'wan:	102° 41'E,	34° 28'N,	3060 m
Datan:	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 seven *Rana kukunoris* study populations in the eastern Tibetan plateau.

ture below 6 °C until before breeding. We randomly collected 10–97 individuals in the ponds or swamps by hand before spawning period (males haven't form a chorus and females are unamplexed), just after their emergence from hibernation at each site. Then, the frogs were identified preliminarily as adult males when the fore digits were discovered bulging brown nuptial pads (SVL > 35mm), others as females (which had large abdomen, indicative of well-developed oocytes; SVL > 40mm). All captured frogs were brought to our field lab near spawning sites. However, at the highest altitude (Guoguo'ri), all the females ran away because the plastic bucket containing the female was broken on the way back to the lab. 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, and body mass (to the nearest 0.01 g) was weighed with an electric balance. Subsequently, frogs were maintained in fresh water for 48 hours to allow full hydration before being euthanised with an overdose of TMS (Tricaine methane sulfonate, CAS: 886-86-2, Purity: > 97.0%, Sigma-Aldrich). The liver and fat bodies were divided and weighed to the nearest 0.1 mg with an electronic balance after placement on water-absorbing paper for around five minutes (Lu, 2004). Finally, the determination of sex and sexual maturity was confirmed by the condition of the gonads at autopsy.

Age determination

We removed the longest phalange of the left hindfoot of adults from 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 counted the number of lines of arrested growth (LAG) in the sections to determine age. Numerous studies have confirmed that improved method of Ehrlich's haematoxylin stain and paraffin section dis-

Table 1. Characteristics of the sample-collecting sites in the east Tibet Plateau, China.

Population	Altitude (m)	Latitude (degrees)	Annual mean temperature (°C)	Hibernation period (days)	Breeding period (days)	Dormancy period (days)	Activity period (days)
Dabaozi'cun	2297	36.65	5.30	152	9	14	190
Shiya'zhuang	2594	36.68	3.50	168	10	17	170
Damoshi'cun	2789	36.49	0.20	196	15	24	130
Gongquhu'cun	3049	34.49	1.50	187	11	17	150
Shibadao'wan	3060	34.47	1.40	191	10	19	145
Datan	3233	34.37	1.00	193	11	21	140
Guoguo'ri	3441	34.29	0.80	193	15	22	135

Table 2. Comparisons of SVL, body mass, age, liver, and fat body of *Rana kukunoris* from seven altitudes in the east Tibet Plateau, China. Values represent mean ± SE for each measure. n = number of individuals.

Population	Sex	n	Collected frogs/ the total frogs (%)	SVL (mm)	Body mass (g)	Age (years)	Liver (mg)	Fat body (mg)
Dabaozi'cun	Female	7	7.29	61.71 ± 1.96	32.07 ± 3.64	4.00 ± 0.31	412.50 ± 91.92	85.66 ± 17.72
	Male	17	17.71	54.70 ± 0.90	17.38 ± 0.86	2.82 ± 0.13	492.86 ± 38.14	90.05 ± 25.48
Shiya'zhuang	Female	13	9.29	58.20 ± 1.35	28.93 ± 2.50	4.00 ± 0.25	201.49 ± 40.84	5.57 ± 2.04
	Male	39	27.86	51.24 ± 0.58	18.35 ± 0.62	3.05 ± 0.10	511.41 ± 23.15	65.91 ± 24.00
Damoshi'cun	Female	44	23.16	56.12 ± 0.61	25.58 ± 0.92	3.48 ± 0.12	376.10 ± 18.75	42.63 ± 6.35
	Male	53	27.89	50.25 ± 0.58	15.39 ± 0.69	2.77 ± 0.09	526.45 ± 30.29	83.28 ± 8.23
Gongquhu'cun	Female	18	26.47	59.82 ± 0.62	32.53 ± 1.26	4.06 ± 0.13	379.56 ± 18.37	8.33 ± 3.25
	Male	19	27.94	54.57 ± 0.59	18.23 ± 0.79	3.32 ± 0.13	586.21 ± 33.28	81.81 ± 10.78
Shibadao'wan	Female	9	21.43	60.42 ± 1.43	31.72 ± 2.10	4.11 ± 0.26	155.60 ± 12.51	3.28 ± 0.74
	Male	11	26.19	51.23 ± 0.90	14.74 ± 0.72	3.18 ± 0.12	421.87 ± 38.01	39.04 ± 7.15
Datan	Female	30	24.59	56.44 ± 0.52	26.82 ± 0.95	3.97 ± 0.12	190.39 ± 13.68	16.02 ± 3.34
	Male	24	19.67	47.40 ± 0.70	12.46 ± 0.41	2.92 ± 0.12	359.83 ± 16.93	44.17 ± 6.25
Guoguo'ri	Male	10	8.26	51.74 ± 098	17.56 ± 0.94	3.20 ± 0.13	515.00 ± 30.10	51.37 ± 7.33

play seasonal growth of amphibian species (e.g., Yu and Lu, 2013; Yu et al., 2019; Yu et al., 2021).

Environmental factor collection

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

Statistical analyses

Prior to analyses, the organ weights, body mass, SVL and age were log transformed to approximately conform to normality. Then, we performed a General Linear Model (GLM) to test whether absolute weights of both liver and fat body differed across populations and between sexes, where population and sex as fixed factors, including an interaction between sex and population. If the overall GLM results were significant, we did pairwise multiple comparisons (post hoc LSD tests) to evaluate differences among populations. To further investigate the differences in relative organ weights across populations and between sexes, we performed a GLM treating weights of liver and fat bodies as dependent variable, population and sex as fixed factors, body mass and age as a covariate. To test the effects of variation in temperature on relative organ weights, we also used GLM treating the organ weights as dependent variables, variation in temperature, body mass and age as a covariate. SPSS 20.0 (SPSS Inc., Chicago, Illinois, USA) was used for all analyses.

RESULTS

Absolute weights of both liver and fat body variation

We detected significant differences in absolute weights of both liver and fat body among populations and between sexes (Table 2, 3). Post hoc LSD tests exhibited those frogs from population Datan, Shibadao'wan and Shiya'zhuang, had respectively lesser absolute weights of both liver and fat body, compared with population of Gongquhu'cun, Dabaozi'cun and Damoshi'cun, respectively (Fig. 2). Males had heavier livers ($t_{292} = 10.824$, $P < 0.001$) and fat bodies ($t_{292} = 11.297$, $P < 0.001$) than females. Moreover, larger individuals had heavier livers ($F_{1,284} = 33.380$, $P < 0.001$), but not heavier fat bodies ($F_{1,284} = 3.021$, $P = 0.083$).

Relative weights of both liver and fat body variation

The significant differences in relative weights of both liver and fat body were observed among populations and between the sexes (Table 4). For female, the population at Shibadao'wan, Shiya'zhuang and Dabaozi'cun, had respectively the smallest relative weights of both liver and fat body whereas frogs from population Gongquhu'cun, Dabaozi'cun and Datan had respectively the heaviest weights of liver and fat body (Fig. 2). For male, relative weights of liver and fat body were the smallest in population Dabaozi'cun, Shiya'zhuang and Shibadao'wan, whereas population Gongquhu'cun, Damoshi'cun and Shiya'zhuang were characterized by the largest weights of liver and fat body, respectively (Fig. 2). Generally, males had relatively heavier livers ($t_{292} = 12.861$, $P < 0.001$) and fat bodies than females ($t_{292} = 8.406$, $P < 0.001$). Moreover, the relative weights of liver in each sex and relative weights of fat body in male were negatively correlated with temperature (Fig. 3; Table 5).

Table 3 The generalized linear model for liver and fat body among the seven high-altitude populations of *Rana kukunoris* along the eastern Tibetan Plateau.

Response variable	Source of variation	df	MS	F	P
Liver	Intercept	1	1112.437	31237.714	< 0.001
	Sex	1	4.172	117.160	< 0.001
	Population	6	0.448	12.569	< 0.001
	Error	286	0.036		
Fat body	Intercept	1	270.373	943.468	< 0.001
	Sex	1	36.575	127.628	< 0.001
	Population	6	2.358	8.227	< 0.001
	Error	286	0.287		

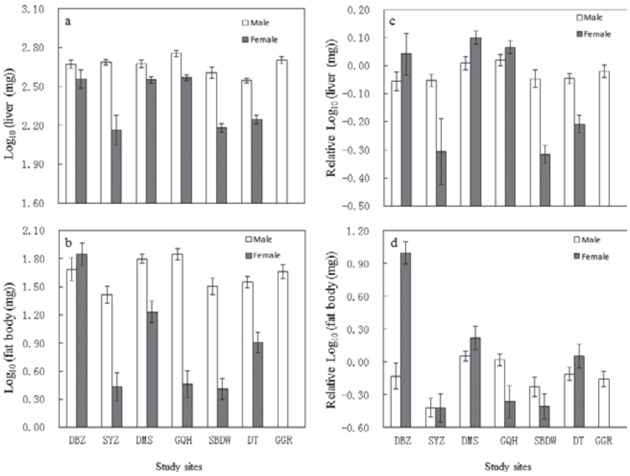


Fig. 2. Absolute liver mass (a), fat body mass (b), relative liver mass (c), and relative fat body mass (d) among seven populations in *Rana kukunoris* (DBZ: Dabaozi'cun, SYZ: Shiya'zhuang, DMS: Damoshi'cun, GQH: Gongquhu'cun, SBDW: Shibadao'wan, DT: Datan, GGR: Guoguo'ri). Relative organ mass represents the residual mean value for a given population corrected for the effect of body size.

DISCUSSION

Anurans living in colder regions usually store more energy after hibernation and before breeding (Jönsson et al., 2009). Our results supported this prediction: when removing the effects of age and body size, liver weights in both sexes and fat body weights in male were negatively correlated with temperature.

Here, larger energy stores in cold environments could be that anurans need chronically plenty of energy

Table 4 The generalized linear model for liver and fat body (with age and body mass as a covariate) among the seven high-altitude populations of *Rana kukunoris* along the eastern Tibetan Plateau.

Response variable	Source of variation	df	MS	F	P
Liver	Intercept	1	5.090	163.409	< 0.001
	Sex	1	5.077	162.986	< 0.001
	Population	6	0.387	12.414	< 0.001
	Age	1	0.004	0.133	0.716
	Body mass	1	1.040	33.380	< 0.001
	Error	284	0.031		
Fat body	Intercept	1	1.022	3.580	0.059
	Sex	1	20.168	70.665	< 0.001
	Population	6	2.209	7.741	< 0.001
	Age	1	0.421	1.473	0.226
	Body mass	1	0.862	3.021	0.083
	Error	284	0.285		

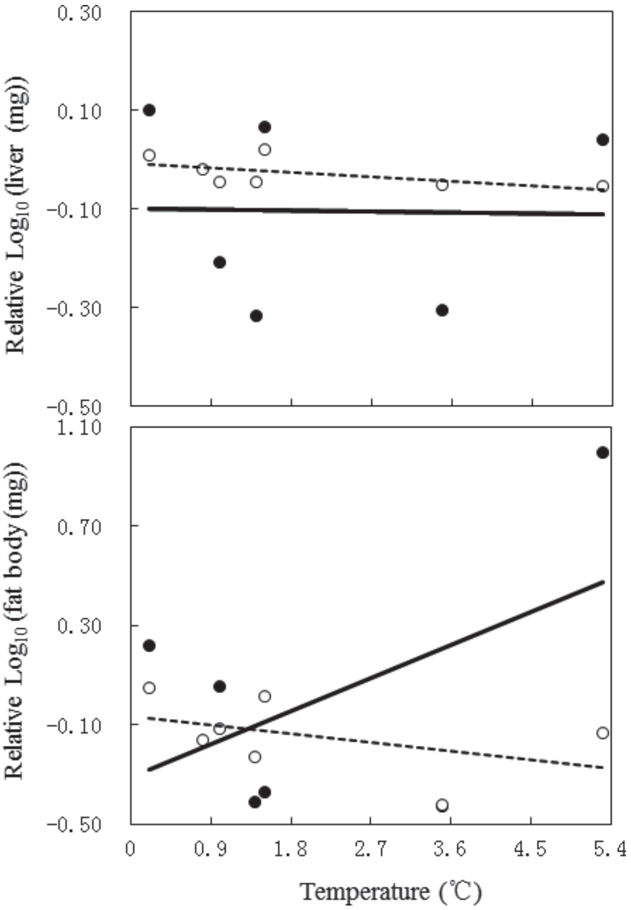


Fig. 3. The effect of annual mean temperature on mean organ mass in males and females from seven populations of *Rana kukunoris* along the eastern Tibetan plateau. Solid and dashed lines are fitted lines for females and males, respectively.

Table 5 The generalized linear model for the effects of temperature on relative organ size of energy storage among the seven high-altitude populations of *Rana kukunoris* along the eastern Tibetan Plateau.

Sex	Response variable	Source of variation	Slope	SE	t	P
Female	Liver	Intercept	1.691	0.293	5.767	< 0.001
		Temperature	-0.042	0.017	-2.467	0.015
		Age	-0.120	0.327	-0.367	0.714
		Body mass	0.588	0.253	2.327	0.022
	Fat body	Intercept	2.332	0.823	2.834	0.005
		Temperature	0.013	0.048	0.276	0.783
		Age	-2.649	0.917	-2.889	0.005
		Body mass	0.065	0.709	0.092	0.927
Male	Liver	Intercept	1.634	0.106	15.363	< 0.001
		Temperature	-0.013	0.006	-2.028	0.044
		Age	-0.165	0.126	-1.310	0.192
		Body mass	0.947	0.103	9.221	< 0.001
	Fat body	Intercept	0.568	0.318	1.790	0.075
		Temperature	-0.072	0.019	-3.767	< 0.001
		Age	-0.127	0.377	-0.338	0.736
		Body mass	1.061	0.307	3.458	0.001

storages to survive the harsh and extended hibernation period. However, *R. kukunoris* enter another short dormancy period after breeding because the environmental condition (e.g., cold temperature) is not suitable for individuals to feed, which could be one possible explanation for more residual energy stores. Further, length of dormancy period decreased with increasing temperature ($r_s = -0.991$, $P < 0.001$, Table 1), indication of longer dormancy period in cold populations. Hence, they must deposit sufficient energy to survive the harsh and extended dormancy period. Additionally, larger energy reserves are necessary for amphibians living with less predictable environmental conditions because more extreme weathers (e.g., temperature plummet) would occur right after their last dormancy (McNamara and Houston, 1990; Jönsson, 1997; Jönsson et al., 2009; Chen et al., 2011a). In accordance with this, Niewiarowski (2001) reported also detected a similar pattern for the eastern fence lizard *Sceloporus undulates* between higher latitude and altitude population. Thus, Chen et al. (2013) suggested that liver weights of *R. kukunoris* in increased high altitudes where indicate low temperature might be an adaptation to the hypoxic aquatic environment in cold regions.

Another possible explanation is allocating more energy for reproduction in cold environments. Due to limited food sources, intense interspecific competition and greater annual mortality risk, females living in cold environments were more likely to allocate more energy to current breeding efforts (Chen et al., 2013). For example, females of *R. kukunoris* living in high-altitude environments favours allocating more energy for maternal investment independent of body size, which cope with the unfavorable and unpredictable weather conditions (Chen et al., 2013). Furthermore, female frogs must utilize stored energy to support early egg development due to lacking of a resting period, indicated that females rely on a single tactic of capital breeding, thus the energy storage should be larger in cold populations. Instead, testis mass of *R. kukunoris* tend to increase with temperature, suggesting that reproductive investment in males is not high in cold environments (Yu et al., 2022). However, we found that the length of breeding period is negatively correlated with temperature ($r_s = -0.909$, $n = 7$, $P = 0.005$, Table 1), indication of longer breeding period in cold populations. We speculated that relatively stable warm temperatures in low altitudes promote synchronous breeding of most toads, resulting in shorter breeding period. In this case, males living at cold environments need more energy reserves to calling and breeding activities in early spring. Therefore, maternal investment in both sexes could partly explain pro-hibernation energy stores increased with decreasing temperature.

Considering the difference between sexes in the timing of the main energy allocation for reproduction, females allocate most of their energy storage in egg development before the mating period, while males put more of their energy investment into their mating efforts during the mating period (Jørgensen, 1981; Jönsson et al., 2009; Chen et al., 2023a,b). Thus, sexual differences in energy stores might be expected after hibernation. Our results showed that males had relatively heavier livers and fat bodies males than females. For capital breeding anurans, in adult females primarily exploit fat bodies to provide energy for the developing gonads (Jørgensen et al., 1979; Jönsson et al., 2009). Here, female *R. kukunoris* finish their main follicular growth before hibernation, and their gonad weight increases before overwintering (Chen et al., 2013), whereas adult males utilize the fat bodies to conduct breeding activities in spring (Yu et al., 2013; Yu et al., 2016; Yu et al., 2018). Thus, males may have more energy reserves prior to breeding than females.

In general, we found that *R. kukunoris* from cold environments had more energy storages after hibernation prior to breeding than individuals from warm environments. Parental investment in both sexes could partly explain pro-hibernation energy stores increased with decreasing temperature. Additionally, these reserves were also used for surviving the harsh and extended dormancy period, indicating of buffering against uncertain environmental conditions. Males had more energy reserves than females due to differences in energy allocation in varied reproductive periods. Therefore, we suggest that a combination of environmental conditions and reproductive roles is the important to analyses energy allocation and reserve patterns in breeding amphibian species.

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