

High Altitude Frogs (*Rana kukonoris*) Adopt a Diversified Bet-hedging Strategy in the Face of Environmental Unpredictability

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Abstract Environmental unpredictability can influence strategies of maternal investment among eggs within a clutch. Models predict that breeding females should adopt a diversified bet-hedging strategy in unpredictable environments, but empirical field evidence from Asia is scarce. Here we tested this hypothesis by exploring spatial patterns in egg size along an altitudinal gradient in a frog species (*Rana kukonoris*) inhabiting the Tibetan Plateau. Within-clutch variability in egg size increased as the environment became variable (e.g., lower mean monthly temperature and mean monthly rainfall at higher altitudes), and populations in environments with more unpredictable rainfall produced eggs that were smaller and more variable in size. We provide support for a diversified bet-hedging strategy in high-altitude environments, which experience dynamic weather patterns and therefore are of unpredictable environmental quality. This strategy may be an adaptive response to lower environmental quality and higher unpredictable environmental variance. Such a strategy should increase the likelihood of breeding success and maximize maternal lifetime fitness by producing offspring that are adapted to current environmental conditions. We speculate that in high-altitude environments prone to physical disturbance, breeding females are unable to consistently produce the optimal egg size due to physiological constraints imposed by environmental conditions (e.g., duration of the active season, food availability). Species and populations whose breeding strategies are adapted to cope with uncertain environmental conditions by adjusting offspring size and therefore quality show a remarkable degree of ability to cope with future climatic changes.

Keywords altitude, bet-hedging strategy, environmental quality, egg size, within-clutch variation, unpredictable environment

1. Introduction

Given finite resources, mothers generally make an energetic trade-off between current reproductive investment and somatic maintenance (Stearns, 1992). This strategy serves to enhance fitness by ensuring future reproductive potential by producing offspring that vary in size and quality (e.g., many small or fewer larger)

based on the environmental conditions of the habitat. The outcome is a balance between the benefits of increased fecundity and high offspring quality (Morrongiello *et al.*, 2012). A mother's best option should be to produce as many offspring of optimal size as is possible, which is adjusted depending on environmental cues (Einum and Fleming, 2004). Such resource allocation within eggs of a clutch is influenced by the environmental variation (Einum and Fleming, 2004; Morrongiello *et al.*, 2012). Empirical support for these patterns is widespread among diverse taxa including plants (Vaughton and Ramsey, 1998), insects (Zovi *et al.*, 2008), fishes (Johnston and

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Leggett, 2002; Morrongiello *et al.*, 2012; Marshall and Burgess, 2015), amphibians (Räsänen *et al.*, 2008), and birds (Tremblay *et al.*, 2003).

In addition to trade-offs between the size and number of offspring produced, breeding females often vary offspring size within a clutch based on the environmental cues they experience throughout ontogeny (Kaplan and Cooper, 1984). When environments vary predictably with the seasons, females are likely to lay eggs that are of optimal size, and thus that are similar within and among clutches (Kaplan and Cooper, 1984). When environments vary unpredictably among years through evolutionary time, females should instead evolve a bet-hedging breeding strategy that increases geometric-mean fitness (Philippi and Seger, 1989; Simons, 2011, 2014). When using a conservative bet-hedging strategy, females may produce offspring that are larger but less variable in size because large eggs may have greater probability of survival in unpredictable environments, as compared to a stable environment (Shine, 1978; Travis, 1980). This strategy can result in greater reproductive success in years when environmental quality is poor, especially if larger offspring have a size-based survival advantage in these conditions (Einum and Fleming, 2004). Alternatively, females can also employ a diversified bet-hedging strategy to produce a range of offspring sizes, which spreads the risk of incorrectly predicting future environmental conditions to ensure at least some offspring survive, regardless of future conditions (Capinera, 1979; Kaplan and Cooper, 1984; Parker and Begon, 1986; Einum and Fleming, 2004; Simons, 2011). According to the imperfect information hypothesis, the degree of within-clutch variability in egg size will decrease relative to average egg size (Koops *et al.*, 2003), and environmental quality also influences the degree of egg size variation by desiccation stress and/or temperature and climate variability (Morrongiello *et al.*, 2012; Marshall and Burgess, 2015; Shama, 2015).

High altitude environments are ideal for testing the predictions of bet-hedging models because of the high degree of environmental variation and unpredictability that occurs over small spatial scales. Animals living at higher altitudes usually face harsher and less predictable environments compared to ones living at lower altitudes (Duellman and Trueb, 1986; Morrison and Hero, 2003; Chen *et al.*, 2013). These environmental conditions should result in population divergence of reproductive strategies along altitudinal gradients that are most suited to local conditions. Most amphibians show variation in life history traits among populations at different altitudes, including

shorter breeding seasons and activity periods, larger eggs and longer juvenile stages, and larger body sizes at all life stages being associated with high altitudes (reviewed by Morrison and Hero, 2003). The shorter and more variable activity periods of high-altitude environments can directly constrain foraging time and therefore reduce energy accumulation (Morrison and Hero, 2003; Chen *et al.*, 2011). Stored energy will determine breeding investment strategies of explosive breeding species, based on an assessment of environmental quality (Wells, 2007). However, it is difficult for selection to optimize any one life history trait in a vicissitudinous environment (Morrison and Hero, 2003), and therefore selection may favor bet-hedging strategies. The Tibetan Plateau, which experiences highly variable seasonal temperature and rainfall patterns, provides an ideal location to test the hypothesis that unpredictable environments select for within clutch variation in egg size as predicted by theoretical work on bet-hedging strategies (McGinley *et al.*, 1987; Einum and Fleming, 2004; Marshall *et al.*, 2008).

We investigated within clutch variation in egg-size of *Rana kukunoris*, a frog species distributed along an altitudinal gradient on the Tibetan Plateau, to test the following predictions: within-clutch variation in egg size will be (1) positively associated with altitude; (2) negatively associated with mean egg size and positively associated with clutch size; (3) negatively associated with environmental quality, and (4) negatively associated with environmental predictability. We also carried out an experiment to investigate the influence of body size on desiccation and survival of tadpoles. Such trade-offs could help understand how maternal breeding strategies are adapted to unpredictable environmental conditions, and therefore provide a glimpse of reproductive tactics that may continue to be successful under future climatic changes.

2. Materials and Methods

Study species The plateau brown frog, *R. kukunoris*, is endemic to high-altitude regions of the eastern Tibetan Plateau, and is one of the few frog species that reaches high elevation (2000–4400 m, Chen *et al.*, 2011). Upon emergence from hibernation in the spring, explosive breeding takes place in shallow temporary ponds, where desiccation and freezing can determine larval survival (Lu *et al.*, 2008). Egg clutches are deposited on the margins of water bodies, which often freeze over during the night (Lu *et al.*, 2008); this, combined with drying of water bodies

during the breeding season can result in larval mortality (Chen, personal observation). After reproducing, females only have a short activity period during which to store energy for the next reproductive bout, prior to ceasing activity for the next winter (Lu *et al.*, 2008; Chen *et al.*, 2011, 2013). Female frogs are the larger sex, and clutch size is positively correlated with female body size, with females at higher altitudes producing larger clutches of eggs (Chen *et al.*, 2013).

Data collection We collected data from six *R. kukunoris* populations (breeding ponds) along the eastern Tibetan Plateau spanning a 255-km latitudinal gradient and ranging in altitude from 2035 to 3494 m above sea level (Figure 1, Table 1). During 2010–2015 we searched for freshly laid egg clutches at breeding ponds. We counted the number of eggs in a small sample of each clutch (ca.

5 g), which was weighed (to 0.01 g) and used to estimate clutch size based on mass of the entire clutch. We quantified egg size by measuring the diameter of 10–30 eggs from digital photographs of each clutch (Abramoff *et al.*, 2004). To ensure that egg size measurements were comparable within and among populations (Räsänen *et al.*, 2008), all clutches were sampled and measured prior to eggs reaching Gosner Stage 12 (Gosner, 1960). We adopted coefficient of variation (CV) in egg diameter as a measure of egg size variation that describes the standard deviation as a percentage of the mean (Quinn and Keough, 2002).

In order to explore the effects of environmental conditions on egg size, we used mean monthly temperature (MMT) and mean monthly rainfall (MMR) to describe environmental quality of each site from



Figure 1 Map showing the distribution of our sampling sites for *Rana kukunoris* in the Tibetan Plateau.

Table 1 Study site details for six high-altitude populations of *Rana kukunoris* in the Tibetan Plateau, including altitude, latitude and sample sizes and clutch attributes.

Altitude (m)	Latitude (degrees)	Number of clutches (<i>n</i>)	Mean egg size (mm)	Std. Deviation	Range (mm)	Egg variation (CV%)
2035	33.16	46	2.07	0.14	1.76-2.44	6.92
3038	34.8	11	2.34	0.12	2.15-2.59	5.3
3061	32.78	45	2.17	0.15	1.91-2.61	7.1
3189	34.1	35	2.02	0.21	1.54-2.49	10.24
3462	34.82	189	1.97	0.13	1.62-2.36	6.66
3493	34.3	84	2.36	0.19	1.98-2.76	7.88

1980 – 2010 (from data. cma. cn). These variables can mirror different aspects of environmental quality. For example, in frogs, MMT is related to activity period and metabolism, whereas MMR can determine food availability and thus potential for energy accumulation and storage (Wells, 2007). For three populations in close proximity to local weather stations (< 20 km), we also used mean monthly rainfall and mean monthly temperature to calculate Colwell's predictability indices for rainfall and temperature (Colwell, 1974).

Tadpole Survival under Desiccation Experimental Design We captured adult frog pairs in amplexus from ponds at an altitude of 3462 m, and took these animals back to Mianyang Normal University. We obtained fertilized eggs by injecting pairs of frogs with synthetic gonadotropins (10 IU/ml chorionic gonadotropin). We measured the egg size and body size of the females, after the eggs were laid.

We used 20 replicates of 10 fertilized eggs each (one from each clutch, Gosner stage 10; Gosner, 1960) of different sizes and placed them into a plastic box (20 cm × 10 cm × 5 cm) containing aged water maintained at 20°C. After 7 days, the numbers of hatched eggs (Gosner stage 18–20) were recorded. At this time, all eggs had either hatched or were clearly inviable. To measure the impact of egg size on larval life, we maintained the tadpoles in the same boxes for two weeks post-hatching with a room temperature of 20°C, and we feed the tadpoles in each box with fish food every three days and change the water every three days. In order to investigate the influence of drought on tadpoles of different size, 40 tadpoles with different body lengths were haphazardly selected to be placed onto dry copy paper, and have survival time recorded as a function of desiccation.

Statistical methods First, we explored the relationships between altitude and egg size variation using General linear models (GLMs) with altitude and latitude as covariates, and CV as the dependent variable. We then investigated the correlation between CV and clutch size as well as CV and mean egg size in another independent

model. To explore the effect environmental quality on egg size, we also explored the relationship between environmental quality (mean monthly temperature and rainfall) and CV using the same models. We explored the relationships between both within-clutch variability of egg size and egg size and environmental predictability using linear regressions using stepwise discriminant function. We explored the correlation between egg size and survival rate, between egg size and tadpole performance, and between body size of tadpoles and their survival rate using the Spearman's correlation coefficients.

Prior to analyses, we log₁₀-transformed all variables to better attain normality and improve homogeneity of variance. All analyses were conducted using IBM SPSS Statistics version 24.

3. Results

Field Data In total, we measured 410 clutches from six populations spanning a 255-km latitudinal gradient and ranging in altitude from 2035 to 3494 m above sea level. Egg size showed significant variation within a clutch (CV range 1.72%–12.73%), among clutches ($t = 56.967$, $df = 409$, $P < 0.001$), and among populations ($F_{5, 404} = 30.357$, $P < 0.001$, Table 1). Body size of females was not correlated with the degree of egg-size variation within a clutch (Spearman's correlation: $r_s = -0.416$, $n = 17$, $P = 0.096$).

The results from GLMs revealed that altitude ($F_{1, 407} = 5.984$, $P = 0.015$), latitude ($F_{1, 407} = 12.659$, $P < 0.001$) can both influence the egg-size variation (Table 2). The degree of egg-size variation did not increase with increasing clutch size ($F_{1, 398} = 1.470$, $P = 0.226$), and this was also the case for mean egg size ($F_{1, 398} = 0.122$, $P = 0.727$), despite significant variation in the degree of egg-size variation among populations ($F_{5, 398} = 31.436$, $P < 0.001$). However, the degree of egg-size variation within a clutch was affected by environmental quality (Table 2), including temperature and rainfall (mean monthly temperature $F_{1, 407} = 57.117$, $P < 0.001$, rainfall $F_{1, 407} = 56.614$, $P < 0.001$). Differences in the estimates of

Table 2 Parameter estimates and test statistics from General linear models describing within-clutch egg-size variation. Model 1 and model 2 are the results testing the altitudinal cline and environmental quality, respectively. CI: Confidence Interval for Mean.

Parameter	<i>B</i>	<i>t</i>	Sig.	95% CI	
				Lower	Upper
Model 1					
Altitude	0.00006	2.44613	0.01486	0.00001	0.00011
Latitude	0.05385	3.55794	0.00042	0.0241	0.0836
Model 2					
Mean Monthly Temperature	-0.19302	-7.55762	< 0.001	-0.24322	-0.14281
Mean Monthly Rainfall	-1.96632	-7.52425	< 0.001	-2.48004	-1.45259

variation were unaffected by sample size variation among populations.

In general, when we considered environmental predictability, only rainfall remained in the final model and rainfall change exerted significant influence on egg size variation in a clutch ($B = -5.769$, $t = -7.267$, $P < 0.001$). Clutch size was not significantly associated with these variables ($B = 0.346$, $t = 0.945$, $P = 0.345$). Females inhabiting more predictable environments laid large eggs ($B = 1.341$, $t = 8.691$, $P < 0.001$) but that were less variable in egg size (Figure 2), whereas females inhabiting more unpredictable environments laid small eggs but more variable egg sizes, subbestinga diversified bet-hedging strategy of energy allocation to offspring.

Desiccation Experiment At Gosner stage 25, the survival rate of tadpoles was not dependent upon initial egg size ($r_s = -0.18$, $n = 20$, $P = 0.941$), but there were obvious differences in the developmental speed of tadpoles; tadpoles from larger eggs grew faster than those from smaller eggs ($r_s = 0.584$, $n = 20$, $P = 0.007$). We also found the large tadpoles from large eggs survived longer than the small tadpoles when in a desiccating environment (Pearson's correlation $r = 0.775$, $n = 40$, $P < 0.001$; Figure 3), indicating that the offspring from large eggs are more likely to survive simulated harsh conditions.

4. Discussion

Altitude is an important environmental factor that affects many life history traits of amphibians, including the duration of the breeding season, size and age at sexual maturity, lifespan, egg number and size, and development and growth of offspring (Morrison and Hero, 2003). Therefore, many life history traits show altitudinal clines in these characteristics (Morrison and Hero, 2003). Environmental unpredictability can also influence strategies of maternal investment among eggs within a clutch (Duellman and Trueb, 1986; Morrison and Hero, 2003; Chen *et al.*, 2013); our study revealed considerable variation in egg size of *R. kukunoris* from Asia, both

within a clutch and among populations differing in altitude and latitude. We also detected an obvious altitudinal cline of egg-size variation within a clutch, but no correlation between egg-size variation within a clutch and mean egg size. Interestingly, our results showed that breeding females produce small, but more variable offspring sizes in unpredictable environments. Similar within-clutch variability of eggs has also been documented in tropical tree frogs from Santa Cecilia, Ecuador (Crump, 1981).

The imperfect information hypothesis (Koops *et al.*, 2003) predicts the degree of within-clutch variability of egg size will be negatively related to average egg size. Our results did not support this prediction because the degree of egg-size variation was not significantly correlated with mean egg size in *R. kukunoris*. We also documented a consistent pattern of females producing a greater range of size variation with eggs, indicating that females are fairly consistent in how they divide resources among eggs. This pattern has also been documented in other animals (Koops *et al.*, 2003; Semmens, 2008).

Our results also provide empirical support for the predictive models developed by Marshall *et al.* (2008), which stipulate that a diversified bet-hedging strategy will occur as an adaptive strategy when future offspring environments are unpredictable. We found that female *R. kukunoris* in these increasingly unpredictable environments produced smaller eggs that are more variable in size. Similar patterns of within-clutch egg size variation with diversified bet-hedging strategy are found across taxa including brook trout *Salvelinus fontinalis* (Koops *et al.*, 2003), common jollytails *Galaxias maculatus* (Semmens, 2008), quacking frogs *Crinia georgiana* (Dziminski *et al.*, 2009) and tree frogs (Crump, 1981). The Tibetan Plateau is a variable and unpredictable environment (Colwell index values 0.89). In this environment, precipitation is an important factor affecting the persistence of habitat and breeding ponds and the survival of offspring, which likely

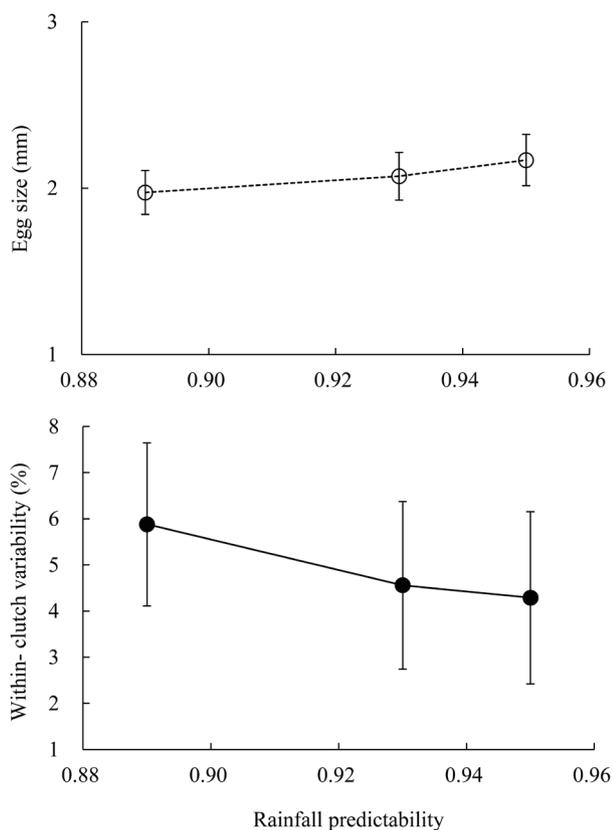


Figure 2 Relationship between egg size and environmental predictability (a) and between within-clutch egg size variation and environmental predictability (b). Environmental predictability is measured using Colwell's index. Error bars represent standard deviation.

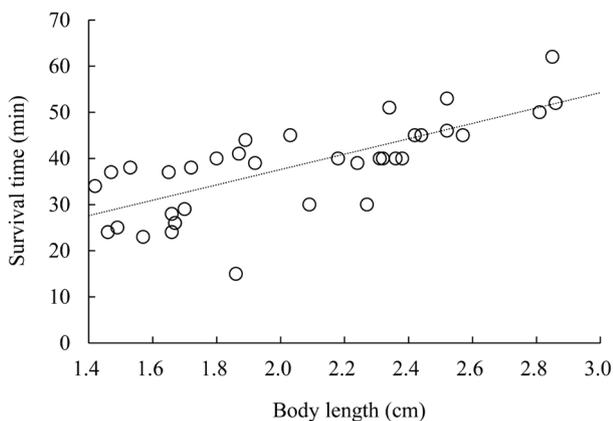


Figure 3 Relationship between survival time and body size of tadpoles in the desiccation experiment, the broken line is the line of best fit.

acts as strong selective forces on the breeding allocation strategies of females. We thus speculate that adopting a diversified bet-hedging strategy may be optimal and adaptive in these low quality high-altitude environments by increasing geometric-mean fitness (Simons, 2011,

2014), because the large tadpoles from larger eggs survive longer in desiccating environments (Figure 2).

Within-clutch variation in offspring size has also been attributed to the physiological or developmental inability of females to precisely allocate resources evenly among individual offspring (McGinley *et al.*, 1987; Fox and Czesak, 2000; Einum and Fleming, 2004), the influence from mothers on offspring environment (van den Berghe and Gross, 1989), or sibling competition (Einum and Fleming, 2002). Even so, we cannot rule out the effects of phenotypic plasticity on egg-size variability, because the concurrent evolution between predictable and unpredictable components in environmental variance is expected (Shama, 2015). In our study, we lack the morphological data on female body condition before oviposition, and were therefore unable to test whether condition influences egg-size variability. Thus the exact causes of egg-size variation within a clutch require further study.

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