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SHORT NOTE

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A comparison of testes size and sperm length between *Polypedates megacephalus* populations at different altitudes

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Variation in temperature and breeding season length caused by altitude has long been considered a major influence on the evolution of life-history traits in amphibians. Here, we examined differences in reproductive investment (testes mass and sperm length) of male spot-legged treefrog (*Polypedates megacephalus*) from two altitudes (680 m and 1300 m above sea level) in Guizhou Province, western China. Relative testes size from the high-altitude population was larger than that of the low-altitude population, whereas sperm length exhibited a converse cline. Testes mass but not sperm length increased with body size at both altitudes. Our findings suggest that differences in reproductive traits between populations might result from differences in the level of sperm competition.

Key words: Altitude, life history, *Polypedates megacephalus*, sperm length, testes size

Relative testis size is widely used as a measure of male reproductive investments, and is considered to be under strong sexual selection (Byrne et al., 2002; Immler et al., 2007; Zhou et al., 2011; Mi et al., 2012; Liao et al., 2013). Males invest more in testes to increase sperm production when the level of sperm competition is high (Dziminski et al., 2009; Liao et al., 2011). In amphibians, a link exists between testis size and environmental factors. For example, relative testes size decreases with increasing altitude and latitude, which is attributed to less energy being allocated to reproduction and/or lowered male intra-sexual competition when environmental temperatures are low breeding seasons are short (Hettyey et al., 2005; Hettyey & Roberts, 2006; Chen et al., 2014; Liao & Lu, 2010; Jin et al., 2016).

Sperm competition occurs if sperm from two or more males compete in fertilising a given set of ova (Parker, 1970). An almost universal adaptation to a high risk of sperm competition is represented by an increased sperm production (Snook, 2005). Sperm size has further been hypothesised to play an important role in determining a male's sperm competitiveness (Parker, 1970; Liao et al., 2013). Depending on the mating system, sperm size is considered to be under stabilising (Calhim et al., 2007) or directional selection (Miller & Pitnick, 2002). Moreover, sperm size might be selected towards different optima across spatially segregated populations (Huey et al., 2000; Blanckenhorn & Hellriegel, 2002), and populations with higher levels of sperm competition are expected to evolve larger testes as well as longer sperm (Lüpold et al., 2011). At increasing latitude and decreasing environmental temperatures, sperm length is largely expected to increase (Huey et al., 2000; Lüpold et al., 2011), although the opposite has also been found (Blanckenhorn & Hellriegel, 2002).

The spot-legged treefrog (*Polypedates megacephalus*) has a wide distribution in China and can be found from 520 to 2200 m in altitude; egg-laying extends from early April to early July (Fei & Ye, 2001). As a rule, between one and five males attempt to mate with a single female; fertilisation of eggs takes place in foam nests, where sperm competition is likely (Liao & Lu, 2011). In the present study, we measure testes size and sperm length at *P. megacephalus* populations from two different altitudes, and explore the relationship between both parameters with operational sex ratio (OSR) and breeding season length.

Field work was conducted in Guizhou Province, western China. The low-altitude population was located at 680 m in Shangzhong (108°43.38'E, 27°23.38'N), and frogs reproduced in a single aquatic site (3.0 × 1.2 m, 0.8 m deep). The high-altitude population was located at 1300 m in the Leigong mountains (108°10.27'E, 26°22.73'N), and frogs reproduced in two ponds (2.0 × 1.5 m, 1.2 m deep, and 12.0 × 4.0 m, 0.3 m deep); both sites were in agricultural areas. Average annual air temperatures were 9 and 16°C in Shangzhong and Leigong, respectively, with a corresponding breeding season length of 85 days and 60 days. A total of 65 adult males were collected by hand during the reproduction peak from 5–12 July 2014. We determined the sex of each individual by phenotypic criteria (presence of nuptial thumb pads in males and the inflated abdomen of females). OSR was recorded for ten nights within each population.

Before processing, male frogs were kept in rectangular tanks (1.0 × 0.5 m) with a water depth of 5 cm at room temperature. Animals were killed by using double-pithing. Body size (snout-vent length: SVL) was measured with a vernier caliper to the nearest 0.1 mm. Testes

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Table 1. Mean and standard deviation of male body size, testes mass and sperm length at two *Polypedates megacephalus* populations.

Characters	High-altitude population		Low-altitude population	
	Mean±SD	Range	Mean±SD	Range
Body size (mm)	43.4±3.3	35.7–49.4	42.9±3.2	34.3–49.1
Testes mass (mg)	91.0±37.5	33.1–207.8	66.2±30.4	26.7–175.4
Sperm length (μm)	212.5±8.1	198.1–245.9	217.6±9.2	189.2–230.3

were removed, weighed to the nearest 0.1 mg using an electronic balance, immediately crushed, and sperm was released into a standard volume of reverse-filtered tap water. A 50 µl sperm suspension was pipetted onto microscope slides to measure sperm length (see Liao et al., 2013). We took digitised photographs of sperm using a Motic BA300 digital camera mounted on a Moticam2006 light microscope at 400x magnification, and randomly chose 9 sperm from each male to measure sperm length using a Linechain tool and the Motic Images Advanced 3.2 software. Sperm length measurements were highly repeatable when three replicate measurements on 585 sperm originating from all males were compared (r=0.94, see Lessells & Boag, 1987).

Differences in body size, testes mass and sperm length between populations were tested using one-way ANOVA. To test for differences in relative testes size between populations, we also ran a GLM treating testes mass as a dependent variable, population as fixed factors, and body size as covariate. To test for differences in sperm length between populations, we applied a further GLM with sperm length as a dependent variable, population as a fixed factor, and body size and testes mass as covariates. The statistical tests were set two-tailed, and the nominal significance level was set at p=0.05.

OSR differed significantly between populations, with a higher male bias at high altitude (Shangzhong: 1.8; Leigong: 3.0; Students t-test: t=7.92, p<0.001). Male body size at the high-altitude population was not significantly larger than that at the low-altitude population (Table 1; one-way ANOVA: $F_{1,63}$=0.325, p=0.571; Table 1). Testes mass was significantly larger in the high- than in the low-altitude population (one-way ANOVA: $F_{1,63}$=7.859, p=0.007; Table 1), also when removing the influence of SVL (GLM; $F_{1,63}$=8.229, p=0.006). Larger males had larger testis mass relative to their body size ($F_{1,63}$=19.607, p<0.001; Fig. 1). Average sperm length was significantly lower at high altitudes (one-way ANOVA: $F_{1,63}$=5.542, p=0.022; Table 1), also when correcting for the influence of body size and testes mass (GLM; $F_{1,63}$=5.410, p=0.023). Variation in sperm length can overall be explained by body size ($F_{1,63}$=5.537, p=0.024; Fig. 2), but not by testes mass ($F_{1,63}$=0.382, p=0.539).

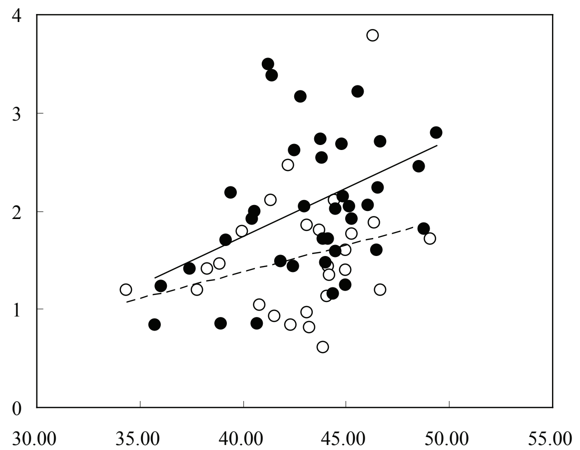

Fig. 1. Relationships between body size and relative testes size in *Polypedates megacephalus* in western China. Closed and open circles represent the high- and low-altitude populations, respectively. Relative testes size=testes mass (mg) / body size (mm).

Previous studies on anurans have suggested that testes mass decreases with altitude and latitude due to decreased competition for mates, shortened time available for reproduction and extended acquisition of resources needed for survival (Hettyey et al., 2005; Chen et al., 2014). In the present study, we found larger testes in *P. megacephalus* in high-altitude population despite a shorter breeding season and lower temperature. Sexual selection is lower at even sex ratios (Wells, 1977), and we thus attribute our finding to the male-biased OSR at high-altitude populations, as a hypothesis increasing the degree of male-male competition.

Females may use an association between sperm traits and body condition for the purposes of sexual selection (Schulte-Hostedde et al., 2004). As a result, relative testis size is often positively related to male body size or condition (Simmons & Kotiaho, 2002). Consistent with most anuran species (Zhou et al., 2011; Mi et al., 2012; Chen et al., 2014), large *P. megacephalus* males had relatively larger testes than small males. However, relative

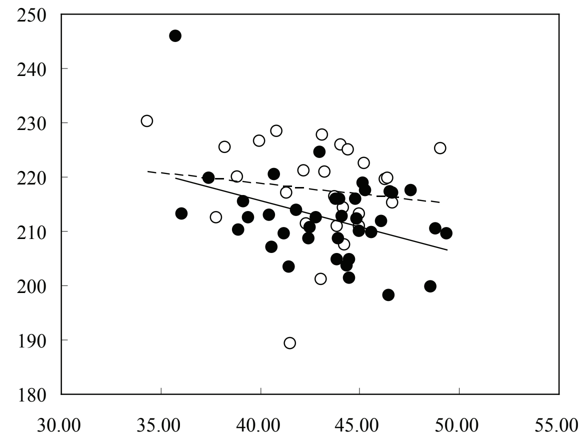

Fig. 2. Relationships between body size and sperm length of *Polypedates megacephalus* in western China. Closed and open circles represent high- and low-altitude populations, respectively.

testis mass in anurans is not necessarily associated with male body condition (*R. omeimontis*: Liu et al., 2012; *Crinia georgiana*: Hettyey & Roberts, 2007).

Interspecific comparisons found that males that invest heavily in sperm produce larger testes and longer sperm to increase their competing ability (butterflies: Gage, 1994; moths: Morrow & Gage, 2000; cichlid fishes: Balshine et al., 2001; amphibians: Byrne et al., 2003; Zeng et al., 2014; birds: Lüpold et al., 2009; mammals: Gage & Freckleton, 2003). Sperm length can however also be negatively correlated with testis mass in frogs (*R. omeimontis*: Liu et al., 2012; *C. georgiana*: Hettyey & Roberts, 2007) and birds (Immler et al., 2007). Sperm length in *P. megacephalus* was uncorrelated with testis mass in both populations. Sperm length can also be negatively related with environmental temperature (Huey et al., 2000; Lüpold et al., 2011), a conclusion which we can confirm with the present study. Whether a shorter breeding season and a more male-biased OSR can lead to a lower intensity of sperm competition or whether increased environmental constraints at high altitude result in smaller sperm requires further study.

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