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Sperm traits in relation to male amplexus position in the Omei treefrog *Rhacophorus omeimontis*, a species with group spawning

Wen Bo Liao[1], Zhi Ping Mi[1], Chen Liang Li[2], Shi Chao Wei[2] & Hua Wu[2]

[1]*Key Laboratory of Southwest China Wildlife Resources Conservation Ministry of Education, China West Normal University, Nanchong, 637009, China,* [2]*Laboratory of Ecology and Evolutionary Biology, College of Life Sciences, Central China Normal University, Wuhan 430079, China*

Sperm competition theory predicts that subordinate males may experience a higher intensity of sperm competition than dominant males if mating tactics are constant, resulting in larger testes, larger sperm and greater longevity. We tested if these predictions can be applied to the polyandrous Omei treefrog (*Rhacophorus omeimontis*). Our results showed that relative testes size did not differ between amplexed males and satellite males, indicating that satellite males might not show signs of higher intensity of sperm competition compared to amplexed counterparts. Sperm size and longevity did not differ significantly between amplexed males and satellite males. Sperm size and longevity were not significantly correlated with each other, and sperm size does not correlate with sperm competition intensity. Our findings suggest that mating position is not related to measures of sperm competition intensity in the Omei treefrog.

Key words: amplexus position, multiple-male mating, *Rhacophorus omeimontis*, sperm competition, sperm size

INTRODUCTION

Sperm competition occurs if sperm from two or more males compete in fertilizing a given set of ova (Parker, 1970). Sperm competition is widely recognized as a widespread and powerful selective force, generating selection on male reproductive anatomy, behaviour and morphology (Birkhead & Møller, 1998; Simmons, 2001). Sperm competition is also thought to be the major force driving the evolution of sperm traits (Parker, 1998; Pizzari & Parker, 2009). An almost universal adaptation to sperm competition in males is represented by increased sperm production in response to increased sperm competition risk, and sperm competition success is frequently determined by relative testes size of rival males (Ball & Parker, 2000; Snook, 2005). Parker (1990) states that if mating tactics are constant, the intensity of sperm competition is higher for subordinate males/sneakers because their sperm will always compete with sperm of dominant males/guarders, whereas this is not always the case vice versa.

Sperm size has been hypothesized to play an important role in determining a male's sperm competitiveness. Selection for enhanced sperm competitiveness favours large sperm in polyandrous species, because larger sperm swim faster and are more likely to win the race to fertilize given eggs (Parker, 1970; Sherman et al., 2008; Lüpold et al., 2009). Moreover, sperm longevity is known to be positively related to sperm competitive success in fish, birds and mammals (Gage et al., 2004; Gomendio et al., 2007; Helfenstein et al., 2010). Besides sperm size and longevity, variation in sperm size appears to be relevant to male success. Several studies have indicated that sperm competition may enforce stabilizing sexual selection on sperm size variation, with high risk of sperm competition being associated with low variation (e.g., Radwan, 1996; LaMunyon & Ward, 1998; Calhim et al., 2007).

The Omei treefrog *Rhacophorus omeimontis* is endemic to mountain ranges in the subtropical forests in western China, where it occurs at altitudes ranging from 750 to 2100 m a.s.l. (Fei & Ye, 2001; Liao & Lu, 2011a). During the breeding season, males gather at ponds and produce advertisement calls to attract females (Liao & Lu, 2010). When females enter the breeding pond, they approach males based on call traits and age (Liao et al., 2011; Liao & Lu, 2011b, c). Amplectant pairs move to a neighbouring plant and produce foam on leaves above the pond. Immediately, other males (hereafter referred to as "joining males") start to interfere with amplectant pairs (Liao & Lu, 2010). The average number of males involved with a single female is 3.4±1.1 individuals per mating (Liao & Lu, 2010), usually joining males as well as pairing males ejaculate in the foam which serves as the medium for fertilization. As a hypothesis, pairing males experience less sperm competition than joining males

Correspondence: Wen Bo Liao (Liaobo_0_0@126.com)

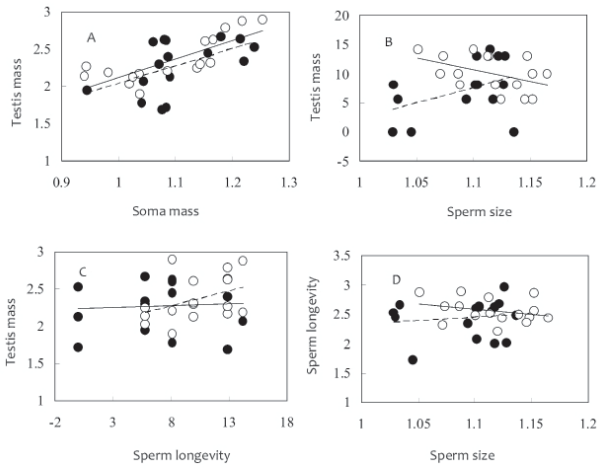

Fig. 1. Correlations between soma mass and testes mass (A), sperm size and testes mass (B), sperm longevity and testes mass (C), and sperm size and sperm longevity (D) in pairing and joining males (full circles: pairing males; empty circles: joining males). Displayed values are transformed data for the ease of interpretation.

because (i) pairing males have longer and better access to the female during egg laying, and (ii) joining males should experience a reduced likelihood of mating with a female at the optimal time of egg fertilization. In the present study, we test the hypothesis that joining males have larger testes relative to their body size compared to amplexed males, and that sperm size and longevity differ between joining and pairing males.

MATERIALS AND METHODS

We studied *R. omeimontis* in a permanent pond (2×1.5×1.2 m) with a depth of 25 cm at Dengchigou Protection Station in western China (102°56'E, 30°33'N, elevation 1700 m). During the 6th and 7th of May 2010 and the 19th and 20th of May 2011, we captured males by hand at night during spawning using a 6V flashlight. The following combinations of pairing and joining males were brought to the laboratory: four pairing males and 5 joining males on 6th May and 4 pairing males on 7th May, 2010, as well as 3 pairing males and 4 joining males on 19th May and 5 pairing males and 7 joining males on 20th May, 2011. Preceding mark-recapture studies showed that, with the exception of a single male, individuals did not switch between the two mating strategies (WBL, unpublished data). Until processing, each male was kept in a wire-netting rectangular container (20×10×15 cm) placed in a tank (90×40×40 cm) with a depth of 10 cm water at room temperature. Two (2010) and 3 (2011) days after capture, body size (SVL) was measured to the nearest 0.1 mm using a caliper. We killed animals by double-pithing. Both testes were removed and weighed to the nearest 0.1 mg using an electronic balance. Testes were 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 size. Fifteen, 30, 60, and 120 min after sperm release we pipetted 50 µl of the sperm suspensions onto microscope slides. The percentage of living sperm was estimated by counting 100 sperm using a microscope at ×400 magnification. We considered the percentage of live sperm after 60 min as a measure of mean sperm longevity because amplexus, foam nest construction and egg deposition normally takes more than one hour (WBL, unpublished data).

Digitized photographs of spermatozoa were taken using a Motic BA300 digital camera mounted on a Moticam2006 light microscope at ×400 magnification. We randomly chose 20 sperm from each male and measured sperm size using a Linechain tool and the Motic Images Advanced 3.2 software. The ratio between sperm head and sperm tail may be an indicator of sperm size in relation to sperm of different developmental stages in the testes. Sperm size measurements were highly repeatable when we compared three replicate measurements on 640 sperm originating from 32 males (R=0.92, see Lessells & Boag, 1987). To further enhance the reliability of sperm size data, we measured the same 20 spermatozoa three times for a single male using the average size in the analysis. As a measure of sperm size variability for each male, we calculated the coefficient of variation as *CV*=SD/mean*100.

Snout-vent length, body mass, testes mass and sperm size were $\log_{10}$-transformed, and survival percentage was arcsine-square-root transformed to achieve normality. Differences in SVL, body mass and *CV* of sperm size between paired and joining males were tested using one-way ANOVA. We used general linear models (GLMs) treating testes mass as a dependent variable and male mating-position category as a fixed factor. Capture date, time from collection and amplexus group were random factors, and soma mass (body mass-testes mass) served as a covariate to assess difference in testes mass between pairing and joining males. To test for differences in sperm size and longevity between pairing and joining

Table 1. Means and standard deviations of body, testes mass, sperm size for two-group types of *Rhacophorus omeimontis* males. Sperm length is also presented on the basis of individual males.

Characters	Pairing males	Range	Joining males	Range
Body size (mm)	62.8±2.9	54.7–67.0	61.4±53.0	54.5–68.8
Body mass (g)	13.2±2.4	8.9–17.7	13.0±3.0	8.9–18.7
Testes mass (mg)	339.9±211.6	49.4–462.2	406.8±204.3	80.1–748.2
Sperm size	12.46±1.03	10.68–13.68	13.07±1.01	11.25–14.20
CV of sperm length (%)	6.46±2.08	3.18–10.49	6.05±1.80	3.81–9.32

males, we applied a GLM with male mating category as a fixed factor, capture date and time from collection as random factors, and testes mass as a covariate. We used Spearman's rank correlation to correlate sperm longevity and sperm length, applying a Bonferroni correction. All values given are shown as mean±SD and all statistical tests were two-tailed.

RESULTS

There were no significant differences in average body size and body mass between pairing and joining males (Table 1; one-way ANOVA: body size, $F_{1, 31}$=0.11, p=0.74; body mass; $F_{1, 31}$=0.42, p=0.52). Testes mass relative to body size did not differ significantly between pairing and joining males ($F_{1, 31}$=1.40, p=0.29). Testes mass did not show a positive relationship with soma mass ($F_{2, 30}$=3.78, p=0.07), capture date ($F_{4, 28}$=1.58, p=0.34) and time from collection ($F_{3, 29}$=0.43, p=0.74). The interaction between time from collection and types of males did not affect male testes mass ($F_{3, 23}$=0.81, P=0.50). The interaction between time from collection and capture date was also non-significant ($F_{6, 21}$=0.94, p=0.36). A positive correlation between soma mass and testes mass was observed in joining males (r_s=0.124, p=0.649), but not in pairing males (r_s=0.676, p=0.004; Fig. 1A) when capture date and time from collection are removed from the model.

The difference in average sperm size between pairing and joining males was not significant, and sperm size varied independently from time from collection and capture date (Table 2). Testes mass did not explain a significant variation in sperm size (Fig. 1B), and all interactions between variables were non-significant (Table 2). Sperm longevity did not differ significantly between pairing and joining males (Table 2). Testes mass did not significantly correlate with sperm longevity (Fig. 1C). Capture date and time from collection did not explain significant variation in sperm longevity, and the interaction between time from collection and types of males was not significant. There was no significant interaction between time from collection and capture date (Table 2). *CV* of sperm size did not differ between pairing and joining males ($F_{1, 31}$=2.43, p=0.13). There was no negative correlation between sperm longevity and sperm size for two-group types of males (Spearman's correlation analysis: pairing males, r_s=0.298, n=16, p=0.263; joining males, r_s=-0.446, n=16, p=0.083; Fig. 1D).

DISCUSSION

Parker (1990) predicted that, when mating tactics are constant, subordinate males will invest more toward sperm than dominant males, to compensate for their reduced likelihood of copulating with a female at the optimal time for fertilization. In our study, joining male frogs did not have larger testes compared to pairing males. This result is different from a fundamental prediction made by sperm competition models (Parker, 1990). However, our results are consistent with previous studies which have shown that small males do not invest more in sperm than large males (*Crinia georgiana*, Hettyey & Roberts, 2007).

In amphibians, numerous studies on sperm competition have focused on externally fertilizing species exhibiting alternative male mating tactics (Roberts et al., 1999). In the Australian *C. georgiana*, dominant males can monopolize females, whereas subordinate males often engage in polyandrous matings. Consequently, dominant males may experience a lower intensity of sperm competition than small males, although subordinate males do not have relatively larger sperms or greater longevity than dominant males (Byrne & Roberts, 2004; Hettyey & Roberts, 2007). Joining *R. omeimontis* males are engaged in polyandrous matings, and should experience a higher intensity of sperm competition than pairing males (Liao & Lu, 2010). However, we did not find that joining males had larger sperm and longer sperm longevity than pairing males. This is inconsistent with the predictions of Parker's sperm competition hypothesis.

Given males employ different mating tactics, they may have different sperm size optima depending on the conditions they face (Parker, 1998). We found that sperm size was not related to relative testes mass and thus did not reflect differences in sperm competition intensity faced (see also Hettyey & Roberts, 2007). Theoretical models concluded that sperm longevity should increase with the intensity of sperm competition (Gage et al., 2004). However, it seems unreasonable to assume that increased sperm longevity in *R. omeimontis* should enhance the competitive ability of sperm, due to the lack of a correlation between sperm longevity and sperm size. Birkhead et al. (2005) hypothesized that sperm competition intensity may select for lower variation in sperm traits. Indeed, a negative relationship between indices of sperm competition intensity and variation in

Table 2. GLMs showing the effect of male type, capture date, collection time and interaction between male type time from collection and male type on sperm size and longevity. Sperm size=sperm tail/sperm head.

Variables	Sperm size	Sperm longevity
Types of males	$F_{1, 31}$=0.08, p=0.79	$F_{1, 31}$=2.77, p=0.17
Capture date	$F_{4, 28}$=1.41, p=0.46	$F_{4, 28}$=2.32, p=0.17
Time from collection	$F_{3, 29}$=1.36, p=0.37	$F_{3, 29}$=0.21, p=0.86
Testis mass	$F_{2, 30}$=0.84, p=0.64	$F_{2, 30}$=1.22, p=0.28
Interaction between time from collection and male type	$F_{3, 23}$=0.64, p=0.46	$F_{3, 23}$=1.37, p=0.28
Interaction between time from collection and capture date	$F_{6, 21}$=0.17, p=0.87	$F_{6, 21}$=1.35, p=0.23

sperm size occurs for example in a passerine bird (Immler et al., 2008, Kleven et al., 2008). For *R. omeimontis*, joining males who face strong sperm competition pressure did not have lower variation in sperm size than pairing males. This might be due to analyzing sperm from a range of developmental stages.

Previous studies have indicated that the correlation between sperm size and sperm longevity is unclear. For example, sperm longevity does not decrease with sperm size in the quacking frog *C. georgiana* (Hettyey & Roberts, 2007). Longer sperm swim more slowly than shorter sperm but live longer in birds (Helfenstein et al., 2008; 2010). In our study, there was no relationship between sperm longevity and sperm size for two groups of males, suggesting that longer sperm do not swim faster via sperm competition and show shorter sperm longevity. However, previous mating histories, which were unknown from our field-collected individuals, can have important effects on sperm traits. Moreover, sperm depletion following matings may influence testes weight (Doyle, 2011). The potential effect of mating history on measurements of sperm traits (sperm longevity) need to be considered in future studies.

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