Sperm competition in frogs: testis size and a 'sterile male' experiment on *Chiromantis xerampelina* (Rhacophoridae)

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The allometric relationship between body mass and testis mass was calculated using data from 16 genera (37 species) of African and Japanese frogs. Having controlled for body mass, the relative testis mass of *Chiromantis xerampelina, Rhacophorus arboreus* and *R. schlegelli* was considerably heavier than predicted (3.8–14.6 times more). All three species have multi-male breeding. Although the result is consistent with sperm competition having selected for increased sperm production in anurans, the phylogenetic distribution of well documented multi-male spawning is confined to the Rhacophoridae. Thus, multi-male mating may have arisen only once effectively reducing the analysis to two data points. However, in the four foam-nesting Rhacophorids whose breeding behaviour has been studied there is also a correlation between relative testis mass and the intensity of sperm competition. This suggests that even within the Rhacophoridae, sperm competition leads to larger testes. Direct evidence for sperm competition in *C. xerampelina* is provided by a 'sterile male' experiment, which shows that peripheral males are capable of fertilizing eggs.

ADDITIONAL KEY WORDS:—Allometry – anurans – multi-male spawning – testis mass.

CONTENTS

INTRODUCTION

Why does testis mass[1] vary so widely among species? Short (1977), looking specifically at the great apes, noted that species with multi-male groups tend to

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[1]Testis mass refers to the combined mass of both testes.

have relatively large testes. He suggested a likely explanation for this relationship, namely that multiple-mating by females leads to sperm competition (Parker, 1970; Harvey & May, 1989). A relationship between testis mass and multiple-mating in the apes was later confirmed using the comparative method to control for body-size effects (Harcourt *et al.* 1981). More generally, inter-specific comparative studies of birds and mammals have shown a similar positive relationship between testis mass and the number of males a female is likely to copulate with in a reproductive cycle (mammals: Kenagy & Trombulak, 1986; primates: Harcourt *et al.* 1981; Harvey & Harcourt, 1984; Møller, 1988a; birds: Møller, 1988b, 1991). In butterflies of the Pieridae and Satyridae families, there is a direct relationship between the mass of ejaculate and polyandry, as well as the production rate of ejaculate and polyandry (Svard & Wiklund, 1989).

In mammals and birds, having corrected for the effect of body size, larger testis mass is associated with: higher sperm production rates; larger sperm reserves; and more sperm per ejaculate (Møller, 1988b, 1989). Sperm competition mechanisms are largely unknown (Birkhead & Hunter, 1990), but theoretical models suggest that numerical superiority is an adaptive strategy for sperm competition (Parker, 1990a,b). The situation has been compared to a lottery, where one's probability of winning (fertilizing ova) is directly related to the number of tickets purchased (sperm ejaculated).

Sperm competition has not been conclusively demonstrated in any species of amphibian (Halliday & Verrell, 1984). Some urodeles possess internal fertilization, store sperm and are inseminated by more than one male (Verrell, 1989). This is clearly a situation in which sperm competition may arise, and there is strong circumstantial evidence for its occurrence in certain urodeles (Verrell, 1989). In frogs, however, the potential for sperm competition appears to be more limited. In most species a single male clasps a female and remains in this position, known as amplexus, throughout spawning. Fertilization is almost always external and the amplexing male releases sperm in synchrony with female oviposition. Sperm competition is only likely to evolve if unpaired males have a reasonable chance of fertilizing eggs, but the amplexing male's proximity to the female probably makes this a rare occurrence.

In a few frogs, however, multi-male breeding has been observed. In the African foam-nesting rhacophorids *Chiromantis xerampelina, C. petersii* and *C. rufescens* (Coe, 1974; Jennions *et al.*, in press) and the Japanese foam-nesting rhacophorids *Rhacophorus arboreus, R. schlegelli* (Kasuya *et al.* 1987; Fukuyama, 1991) and *Polypedates leucomystax* (Feng & Narins, 1991) unpaired, peripheral males gather around amplexing pairs. Peripheral males insert their cloacae into the foam during spawning and, at least in *C. xerampelina*, even attempt to juxtapose their cloacae with that of the female during bouts of oviposition (Jennions *et al.*, in press). Peripheral males in these species almost certainly shed sperm, although direct confirmation of this is lacking. Here we provide direct experimental evidence that male *C. xerampelina* can fertilize eggs from a peripheral position.

In species with internal fertilization, there can be selection for mechanisms of sperm precedence and removal (Birkhead & Hunter, 1990). With external fertilization, however, the only mechanisms whereby males can raise their fertilization success are: (1) to increase the number of sperm released and (2) to maintain proximity to the female (Gross, 1985). There should thus be strong selection for increased sperm production, leading to relatively large testes.

Here we analyse our data on testis mass in 18 species of African frogs and data from 19 species of Japanese frogs (collected by Kusano *et al.* 1991). Kusano *et al.* (1991) reported relatively large testis size in two multi-male breeding rhacophorids and suggested that the cause was sperm competition. The allometric relationship between body mass and testis mass was, however, calculated using species as independent data points. This may have led to erroneous conclusions because of the confounding effect of phylogeny (Harvey & Purvis, 1991). Using generic data, we confirm that the testes of species with multi-male breeding are much larger than expected based on their body size.

METHODS

A 'sterile male' experiment

Experiments were performed between December 1991 and January 1992 at a dam on Vienna Game Farm, Hoedspruit, South Africa (24 17' S, 30 58' E). In *C. xerampelina* peripheral males gather around the amplexing pair during oviposition. The female releases an oviductal secretion which she churns into a foam nest, and oviposition occurs during bouts of churning (Jennions *et al.*, in press). Nests are built on branches overhanging water. We located nesting pairs shortly before oviposition commenced and placed the amplexing male's lower body in a plastic bag (20 × 20 cm). The bag was then attached to the tree branch with tape. Peripheral males were allowed to locate the pair and then position themselves on either side of the amplexing pair. They were able to move underneath the bag and remain in contact with the female's cloaca. Two control trials were conducted, during which all peripheral males were removed from the nest. One trial was conducted in which the four peripheral males that arrived at the nest were allowed to remain. Plastic buckets were suspended beneath nests to collect emerging tadpoles, who were then counted. Finally, nests were dissolved and unfertilized eggs were also counted.

Testis mass

We collected specimens during the peak breeding season (December to January) in 1990–1991 and 1991–1992. They were kept in captivity and starved for one to two days to minimize the effect of stomach content on body mass measurements. The wet mass of the body was then measured to the nearest 0.01 g on an electronic balance. Testes were then dissected out and their combined mass measured to the nearest 0.1 mg. 'Testis mass' refers to the combined mass of both testes.

Comparative studies are often confounded by phylogenetic effects, and it is inadvisable to treat species as independent data points. Recommended comparative methods are either directional comparisons, where ancestral traits are compared with descendant traits; or non-directional comparisons, where traits of immediate descendants of a common ancestor are compared with each other (Harvey & Purvis, 1991). Unfortunately, cladistic studies of African anuran phylogenies are in their infancy and a suitable phylogeny is unavailable. In its absence, an alternative approach is to use taxonomic classifications (such as genera, families and orders) as an approximate evolutionary tree (Harvey & Purvis, 1991). Here we pursue this approach, examining the relationship

between testis mass and body mass at the generic level. Organ size usually scales allometrically with body size (Calder, 1984) and we therefore analysed the testis mass/body mass relationship using a linear regression of log-transformed data. Generic points were calculated using the means of the logs of both variables. There are measurement errors for both variables and a reduced major axis analysis is recommended (Harvey, 1982). The size of measurement error is, however, unknown and a Model I regression is thus equally acceptable (Møller, 1991). The correlation coefficient between the variables is high, so the results should converge on those from a reduced major axis regression (Møller, 1991).

Some species were represented by only a few specimens. To test whether sample size affected our measure of relative testis size we ran a step-wise multiple regression (Statgraphics 5.1). The dependent variable was relative testis mass, the predictor variables were: body mass, testis mass (both log-transformed) and sample size. Body mass and testis mass entered the initial model. The addition of sample size into the model did not explain a significant proportion of the residual variation.

RESULTS

The 'sterile male' experiment

In both controls, where peripheral males were excluded from the nest, fertilization success was zero and no tadpoles emerged. Unfertilized egg counts for the control nests were 1670 and 1516. In the experimental trial where peripheral males were present, tadpoles did emerge. Five hundred and twenty tadpoles left the nest, while 896 eggs went unfertilized (hatching success $= 36.7\%$). Plastic bags appeared to be effective condoms, so these tadpoles were sired by peripheral males.

Testis mass in African anurans

We measured the testis mass of 18 species, from ten genera and four families. Data on body mass and testis mass are shown in Table 1. The range of values for testis mass as a percentage of body mass was relatively small $(0.1-0.6\%)$ across 17 of the species, despite a thirty-fold difference in body size between the smallest and largest species. *Chiromantis xerampelina* males had exceptionally large testes that were, on average, 7.8% of body weight (range $= 4.9-11.3\%$, $n = 12$). There was a significant positive relationship between body mass and testis mass $(r = 0.80,\ n = 18$ species, $P = 0.0001)$. When *C. xerampelina* was excluded, the relationship was even stronger $(r = 0.92,\ n = 17$ species, $P < 0.0001)$.

Body mass and testis mass

We plotted the relationship between the mean log of body mass and the mean log of testis mass for 16 genera (Fig. 1) (For data on Japanese species see Kusano *et al.* 1991.) The Model I regression equation was:

Log [testis mass (mg)] =
-4.18 (SE $= 1.08$) $+ 1.46$ (SE $= 0.29$).Log [body mass (mg)]
$[F_{1,15} = 1213.0,\ P < 0.001,\ R^2 = 64.8\%]$

TABLE 1. Body mass and testis mass for 18 species of African anuran. Mean and standard deviation are shown. Relative testis size is the ratio of observed mass to that predicted on the basis of the allometric regression for 16 genera. Data on Japanese species can be found in Kusano *et al.* (1991).

Families	n	Body mass (g)	Testis mass (mg)	Testis/body (%)	Locality	Relative testis size
Hyperoliidae						
Afrixalus fornasinii	11	2.56±0.49	3.8± 0.8	0.15±0.03	Zululand	0.60
Hyperolius marmoratus	20	1.35±0.37	2.9± 1.4	0.21±0.07	Hoedspruit	1.16
H. tuberilinguis	18	1.41±0.27	5.1± 2.5	0.35±0.12	Zululand	1.96
H. argus	20	2.00±0.22	4.1± 1.1	0.21±0.06	Zululand	0.95
Kassina senegalensis	3	5.00±1.31	32.1±17.6	0.62±0.22	Hoedspruit	1.92
K. maculata	11	15.57±2.69	70.6±27.6	0.44±0.12	Zululand	0.82
Rhacophoridae						
Chiromantis xerampelina	12	13.43±2.69	1030±340.7	7.79±2.38	Hoedspruit	14.55
Bufonidae						
Bufo maculatus	11	9.81±1.27	37.3±11.9	0.39±0.14	Venda	0.84
B. gutturalis	9	38.70±6.48	118.7±38.1	0.31±0.08	Venda, Hoedspruit	0.36
B. garmani	5	42.71±5.73	55.5±19.6	0.13±0.06	Hoedspruit	0.17
Ranidae						
Hemisus marmoratum	8	3.16±0.50	3.9± 1.5	0.13±0.04	Hoedspruit	0.46
Tomopterna cryptotis	3	6.54±0.50	18.0± 9.0	0.27±0.15	Hoedspruit	0.72
Leptopelis natalensis	3	4.70±0.76	9.1± 2.0	0.19±0.04	Zululand	0.60
Ptychadena anchietae	10	5.63±0.85	15.2± 1.9	0.28±0.05	Hoedspruit	0.77
P. oxyrhynchus	4	14.85±3.18	31.3± 4.3	0.22±0.04	Zululand	0.39
P. porosissima	3	5.49±0.36	9.5± 8.5	0.17±0.15	Zululand	0.50
P. mascareniensis	3	10.40±0.89	28.4± 0.1	0.19±0.04	Zululand	0.48
Phrynobatrachus natalensis	3	2.58±0.17	2.8± 0.5	0.11±0.02	Zululand	0.44

The slope of the regression is significantly larger than two-thirds (ANOVA $F_{1,14} = 7.66$, $P = 0.015$), but does not differ significantly from unity (ANOVA, $F_{1,14} = 2.59$, $P = 0.13$). Even when the regression is recalculated excluding the two genera with enlarged testis (*Chiromantis* and *Rhacophorus*) the slope remained greater than two-thirds (ANOVA, $P = 0.009$). This result is unexpected, as an allometric scaling factor of two-thirds is commonly found for body organs (Calder, 1984). As an indirect test of the reliability of the regression equation we compared the equations based on species, genera and families. There was no significant difference in the slopes of these equations (ANCOVA, $F_{2,53} = 0.84$, $P > 0.5$, two-tailed) or their elevation (ANCOVA, $F_{2,55} = 0.042$, $P > 0.5$, two-tailed) (Zar, 1984: 300–304).

To remove the effect of body size, we calculated the relative testis mass for individual species, which was the ratio of the observed testis mass to that predicted by the generic regression (Harvey & Harcourt, 1984: Table 3.1). Mean relative testis mass for *Chiromantis xerampelina* was 14.55, for *Rhacophorus arboreus* 10.30, *R. schlegelli* 3.81, *R. viridis* 2.36, *R. owstoni* 2.40 and *Polypedates leucomystax* 1.74. Relative testis size for the other 31 species ranged from 0.17 to 1.96, with the majority (74%) falling below 1.00. Mean relative testis size for foam-nest building rhacophorids was 5.86 (SD = 5.30, $n = 6$), and for the other species it was 0.76 (SD = 0.50, $n = 31$). This difference is highly significant (Mann-Whitney U-test, two-tailed, $P = 0.0002$). Four of the six foam-nest building rhacophorids are known to have multi-male breeding.
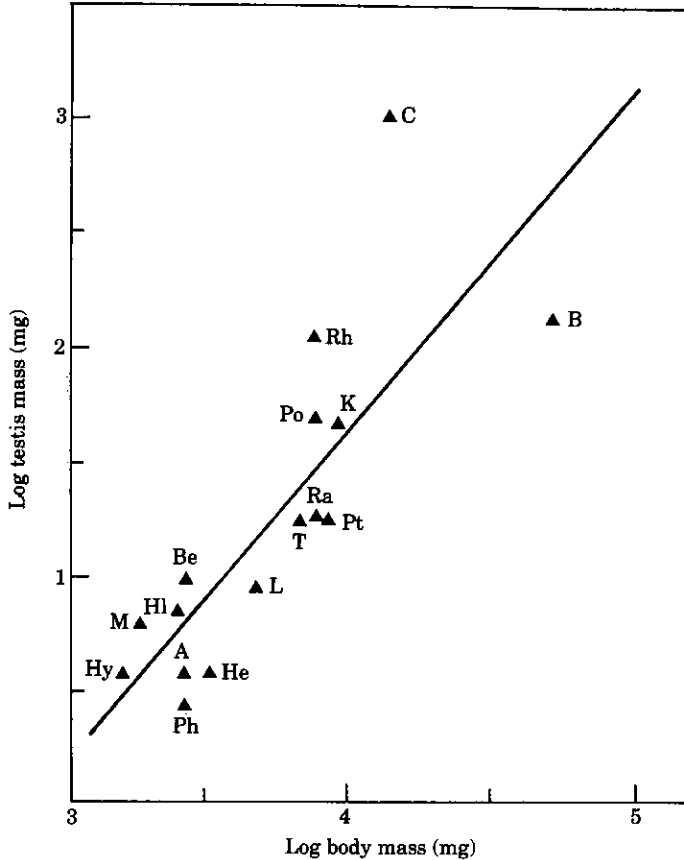
Figure 1. The allometric relationship between body mass and testis mass for 16 genera of anurans. The regression equation is: Log (testis mass) = $-4.18 + 1.46$.Log (body mass) ($F_{1,15} = 1213.0$, $P < 0.001$, $R^2 = 64.8\%$). (Symbols: A = *Afrixalus*, Be = *Buergeria*, B = *Bufo*, C = *Chiromantis*, He = *Hemisus*, Hl = *Hyla*, Hy = *Hyperolius*, K = *Kassina*, L = *Leptopelis*, M = *Microhyla*, Ph = *Phrynomerus*, Po = *Polypedates*, Pt = *Ptychadena*, Ra = *Rana*, Rh = *Rhacophorus*, T = *Tomopterna*).

DISCUSSION

Sperm release by peripheral males

To our knowledge, this is the first study of an anuran to successfully document fertilization of eggs by non-amplexing males. The data conclusively show that peripheral males in *Chiromantis xerampelina* are releasing sperm. Multiple-paternity due to sperm competition, however, may still be absent if the amplexing male has complete priority in access to unfertilized eggs. This is unlikely, but further research is needed. The significance of the low hatching success is unclear. The plastic bag may have affected the rate at which peripheral males came into close contact with the female's cloaca. Even so, this should not have affected the peripheral males' ability to release sperm directly into the foam, suggesting that the juxtapositioning of cloacae with the female may increase fertilization success. Future studies should examine sperm mobility in the foam nest.

TABLE 2. Variation in the frequency and intensity of multi-male breeding in four foam-nesting rhacophorids

Species	Frequency of multi-male breeding (n = females)	Mean number of males participating in a spawning	Maximum number of males participating in a spawning	Relative testis mass
C. xerampelina	92.3% (n = 39)	5.5 (SD = 2.8)	11 a	14.55 (n = 12) (this study)
R. arboreus	81.4% (n = 97)	3.4 (SD = 1.9)	12 b	10.30 (n = 22) d
R. schlegelli	44.4% (n = 9)	1.9 (SD = 1.3)	4 c	3.81 (n = 27) d
P. leucomystax	Seen once	?? e	?? e	1.74 (n = 3) d

(a) Jennions *et al.*, (in press): (b) Toda (1989); (c) Fukuyama (1991); (d) Kusano *et al.*, (1991); (e) Feng & Narins (1991).

Inter-specific variation in testis mass

This study, using a regression based on generic values, confirms the trend that Kusano *et al.* (1991) noted for Japanese anurans, whereby foam-nest building species, which also tend to be multi-male breeding, have relatively large testes. We found that *Chiromantis xerampelina* testes were, relatively speaking, even larger than those of *Rhacophorus arboreus*. Kusano *et al.* (1991) have already outlined an argument for the existence of sperm competition in *R. arboreus* based on testis mass, noting that there is no evidence that the heavy testis mass is related to secretion of substances used in nest construction. In *C. xerampelina*, females add additional foam to the nest the night following spawning and this does not require the assistance of males. There is also no evidence that males help to churn the foam in either species (Kusuya *et al.* 1987). The simplest explanation for the presence of peripheral males is provided by a sperm competition hypothesis.

Testis mass and the frequency of multi-male breeding

Multi-male breeding is confined to foam-nesting rhacophorids from three genera, raising the possibility that the relationship between testis mass and multi-male breeding is spurious. It is possible that multi-male breeding has evolved only once in the rhacophorids. Hence, the relationship between testis mass and multi-male breeding may be the result of a single evolutionary event in an ancestral species. If so, we would not expect a correlation within the group between the level of sperm competition and testis mass. However, if sperm competition does account for variation in testis mass, there should be a positive relationship between the frequency of multi-male breeding, the average number of males participating and relative testis mass, Table 2 shows these data for four species in which multi-male spawning has been observed.

The data indicate that multi-male breeding occurs more often *Chiromantis xerampelina* than in *Rhacophorus arboreus* (92.3% versus 81.4%; although this difference is not statistically significant: Chi-square test, $\chi^2 = 2.5$, $P > 0.05$). Multi-male breeding is also more intensive in *C. xerampelina* (5.5 males versus 3.4 males at a spawning). Multi-male breeding occurs significantly more often, and is more intense, in *R. arboreus* than in *R. schlegelli* (Kusano *et al.* 1991). There is only a single report of multi-male breeding in *Polypedates leucomystax* suggesting

that it occurs infrequently. Relative testis mass is perfectly correlated with the frequency of multi-male breeding and the number of males participating at nests (relative testis mass in: *C. xerampelina* > *R. arboreus* > *R. schlegelli* > *Polypedates leucomystax*) (Table 2), providing support for a sperm competition hypothesis. It should be noted, however, that both *R. viridis* and *R. owstoni* have relatively larger testes than *P. leucomystax*. We can thus predict that they have multi-male breeding. If this is shown, the increased sample size will allow for a rigorous statistic analysis of the relationship between relative testis mass and sperm competition within Rhacophorids. At present, however, there is little data on their breeding behaviour (Kusano *et al.* 1991).

Several factors have been implicated in the high level of multi-male breeding in *R. arboreus* (Kusano *et al.* 1991), and by implication *C. xerampelina:* (1) arboreal nests are more conspicuous and easily located; (2) spawning takes a long time; (3) the sex ratio is strongly male-biased; (4) foam-nesting allows sperm to be contained in the vicinity of the eggs. However, many species with conspicuous foam nests, prolonged amplexus and strongly male-biased sex ratios, do not exhibit multi-male breeding. At best, the combination of these factors may increase the likelihood that multi-male breeding occurs.

Of the eight reported cases of multi-male breeding in anurans, six are from foam-nesting rhacophorids. Furthermore, in the two hylids (*Agalychnis callidryas* and *Phyllomedusa dacnicolor*) (Pyburn, 1970) the frequency of multi-male breeding is very low, and may simply be a consequence of extended attempts at amplexus displacement (personal observations of *A. callidryas*). In the absence of a detailed phylogeny it is impossible to say whether multi-male breeding in foam-nest building rhacophorids is a shared, derived character or has evolved more than once. Those aspects of breeding behaviour, or physiology, which have resulted in the evolution of a reproductive tactic involving sperm competition need to be investigated further, preferably in conjunction with a reliable phylogeny.

Sperm depletion

Sperm depletion provides an alternative explanation for large testis size (Catar, 1985). Van den Berghe & Warner (1989) have documented large testis size (>5% body mass) in group-spawning male coral reef fish (*Thalassoma bifasciatum*). They also noted that sperm competition among group-spawning males appears to limit the number of effective matings a male can engage in. There is no evidence that multi-male breeding Japanese rhacophorids participate in spawning more frequently than species without multi-male breeding (Kusano *et al.* 1991). A study of *Chiromantis xerampelina*, however, showed that males participated in a mean of 3.8 spawnings over a three week period (*n* = 30 males) (Jennions *et al.*, in press). One male participated in at least seven nests. More importantly, several males were involved in spawnings for three or four consecutive nights. A brief survey of the literature on breeding behaviour suggests that this is an exceptionally high level of reproductive activity for an anuran. Sperm depletion has not been documented in naturally breeding anurans, although it has been reported in a laboratory experiment (Smith-Gill & Berven, 1980), but it may be a limiting factor for *C. xerampelina*. Sperm depletion due to increased opportunities to engage in breeding combined with

intense sperm competition may both select for enlarged testis size in *C. xerampelina*.

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