Testes size and breeding systems in Japanese anurans with special reference to large testes in the treefrog, *Rhacophorus arboreus* (Amphibia: Rhacophoridae)

Tamotsu Kusano[1], Mitsuhiko Toda[2], and Kinji Fukuyama[3]

[1] Department of Biology, Faculty of Science, Tokyo Metropolitan University, Minami-ohsawa 1-1, Hachioji, Tokyo 192-03, Japan
[2] Department of Environmental Biology and Health Science, Kanazawa University, Marunouchi 1-1, Kanazawa 920, Japan
[3] Department of Biology, Keio University, Kohoku-ku, Yokohama 223, Japan

Summary. Testes size was compared among 19 species of Japanese anurans in relation to their breeding systems. Although the mean body mass of the species examined varied markedly between 1.8 and 116 g, the mean proportion of testes mass to body mass was fairly constant at 0.2 to 0.4% across all species except the rhacophorid species. Foam-nest building rhacophorids had relatively large testes constituting more than 1% of their body mass. Among them, *Rhacophorus arboreus* had the largest, exceeding 5% of the body mass. Multi-male breeding, where a female is grasped by two or more males during spawning, occurs frequently in these rhacophorids, especially in *R. arboreus*. This close association between large testes size and multi-male breeding strongly suggests that sperm competition is an important factor affecting the evolution of relative testes size in Japanese anurans.

Introduction

In mammals, recent comparative studies have shown that a clear relationship exists between testes size and breeding systems (see Harcourt et al. 1981; Harvey and Harcourt 1984; Kenagy and Trombulak 1986). In multi-male species where females are frequently mated by more than one male during estrus, males generally have larger testes for their body size than single-male or monogamous species where females are rarely mated by more than a single male. Sperm competition is considered to be important in understanding the evolution of relative testes size in mammals, because the sperm competition hypothesis successfully predicts the relationship between testes size and breeding systems. Under the situations where sperm competition occurs, males that produce a large number of sperm relative to other males may have a higher probability of fertilizing the eggs of a particular female. Large testes are, therefore, favored by selection in species with a multi-male breeding system (Harcourt

et al. 1981; Harvey and Harcourt 1984; Kenagy and Trombulak 1986).

In most species of anuran amphibians, a single male grasps a female dorsally in amplexus, and this position is maintained until the female spawns. The male sheds sperm onto the eggs just after spawning. Sperm competition has not yet been demonstrated in any species of anurans (see Halliday and Verrell 1984). Although sperm competition may occur rarely among anurans, some species are known to exhibit multi-male breeding where a spawning female is grasped by two or more males, for example in Mexican leaf frogs, *Phyllomedusa* (Pyburn 1970), and African treefrogs, *Chiromantis* (Coe 1974). In Japan, we also have some multi-male rhacophorid species such as *Rhacophorus arboreus*. In this species, the female is frequently grasped by three to four males, forming a spawning aggregation, as she constructs a foam nest on a tree branch and lays her eggs within the foam. Then, all males participating in the aggregation release sperm into the foam nest (Kato 1956). Such a breeding habit suggests intense sperm competition, and testes size in multi-male species such as *R. arboreus* arouses our interest. However, relative testes size of anurans has not yet been studied in relation to breeding systems, although Iwasawa (1954) suggested that testes size in *R. arboreus* is large compared with a few other species in Japan. In this paper, we present data on testes size and body size for Japanese anuran species and demonstrate that *R. arboreus* has markedly large testes relative to body size. The relationship between testes size and reproductive ecology, including breeding systems, is also discussed from the viewpoint of sperm competition.

Methods

In some species of temperate anurans, testes exhibit seasonal fluctuations in size (Lofts 1974; Rastogi et al. 1986; Long 1987). Breeding males of Japanese anuran species were thus collected during the peak breeding seasons of 1986–1990 to adjust for the reproductive state, except for early breeding species such as *Rana sakuraii*,

Table 1. Body mass and testes mass (combined mass of both testes) for 19 species of Japanese anurans. Means and standard deviations are shown

Families and species	N	Body mass (g)	Testes mass (mg)	Testes/body (%)	Locality
Bufonidae					
Bufo japanicus	4	94.09 ± 19.08	331.75 ± 173.53	0.35 ± 0.15	Ishikawa
B. torrenticola	2	97.64 ± 18.97	306.00 ± 50.91	0.31 ± 0.01	Ishikawa
B. marinus	3	115.67 ± 19.69	344.33 ± 49.14	0.30 ± 0.06	Chichijima Island
Hylidae					
Hyla japonica	10	2.49 ± 0.31	6.85 ± 2.16	0.28 ± 0.10	Chiba
Ranidae					
Rana tsushimensis	2	3.23 ± 0.36	6.28 ± 2.31	0.19 ± 0.05	Tsushima Island
R. tagoi	14	5.88 ± 2.70	23.19 ± 11.89	0.45 ± 0.26	Tokyo, Chiba
R. sakuraii	11	8.98 ± 1.00	21.30 ± 5.66	0.24 ± 0.05	Tokyo
R. chensinensis	6	6.52 ± 2.14	19.60 ± 7.70	0.30 ± 0.06	Hokkaido
R. ornativentris	9	13.81 ± 3.77	36.05 ± 13.41	0.26 ± 0.05	Tokyo
R. porosa	9	14.94 ± 3.92	24.82 ± 8.99	0.17 ± 0.05	Tokyo, Chiba
R. rugosa	5	6.73 ± 1.69	14.65 ± 5.53	0.22 ± 0.05	Chiba
Rhacophoridae					
Rhacophorus arboreus[a]	22	13.89 ± 4.59	753.86 ± 427.93	5.15 ± 2.00	Chiba, Shizuoka, Ishikawa
R. schlegelii[a]	27	3.21 ± 0.55	33.45 ± 10.88	1.06 ± 0.35	Chiba
R. viridis[a]	21	9.12 ± 3.05	96.33 ± 38.52	1.11 ± 0.44	Okinawa Island, Amami Island
R. owstoni[a]	3	7.56 ± 1.01	73.13 ± 29.10	0.95 ± 0.27	Ishigaki Island, Iriomote Island
Polypedates leucomystax[a]	3	7.22 ± 1.12	49.67 ± 36.30	0.71 ± 0.54	Okinawa Island
Buergeria buergeri	10	4.09 ± 0.50	21.20 ± 5.21	0.52 ± 0.14	Chiba, Kanagawa
B. japonica	19	1.79 ± 0.26	4.28 ± 1.61	0.25 ± 0.08	Okinawa Island, Amami Island
Microhylidae					
Microhyla ornata	12	1.77 ± 0.20	6.18 ± 1.83	0.35 ± 0.09	Okinawa Island

[a] Species constructing foam nests

R. tagoi, and *Bufo japonicus.* Since in early breeding species such as *B. japonicus* testes already become largest in autumn (Iwasawa 1987), males were collected late in that season. They were killed with ether, fixed in 10% formalin, and preserved in 70% ethanol. The wet mass of the body and testes of each specimen was determined to the nearest 1 mg and 0.01 mg, respectively, after being sufficiently blotted. These measurements were usually made within a few days after collection.

Results

Testes size of Japanese anurans

A total of 34 anuran species belonging to eight genera of five families are known to exist in Japan (Maeda and Matsui 1989). Among them, we measured testes size in 19 species including seven genera in five families. Body mass, testes mass (combined mass of both testes), and the proportion of testes mass to body mass in each species are shown in Table 1. Although the mean body mass of species examined varied markedly from 1.8 to 116 g, the mean proportion of testes mass to body mass remained fairly constant at 0.2 to 0.4% across all species except the rhacophorid species. Some rhacophorids had relatively large testes; that is, the testes weighed, on aver-

age, more than 1% of the body mass (*R. arboreus, R. schlegelii,* and *R. viridis*). Among them, the testes of *R. arboreus* were especially large, weighing more than 750 mg and exceeding 5% of the body mass (Table 1).

To compare body size-related organ size among species with wide ranges of body size, we must consider allometric relationships between organ size and body size (Harvey and Harcourt 1984). Mean testes mass in each species is plotted against mean body mass on a logarithmic basis (Fig. 1). It is shown that a significant positive correlation exists between body mass and testes mass ($r = 0.83$, $N = 19$, $P < 0.001$); that is, larger species tend to have larger testes. All foam-nest building rhacophorid species, however, have large testes relative to body size, as compared with species belonging to other families and the two rhacophorid species of *Buergeria* that do not construct foam nests. To remove the influence of body size, relative testes size was calculated as the ratio of observed testes mass to the testes mass predicted by the regression equation shown in Fig. 1. Mean relative testes size of foam-nest building rhacophorids was 4.52 (SD = 4.98, $N = 5$), ranging from 1.69 to 13.41; that of other species was 0.72 (SD = 0.22, $N = 14$), ranging from 0.41 to 1.27. This difference is highly significant (Mann-Whitney U-test, $U = 0$, $P < 0.01$).
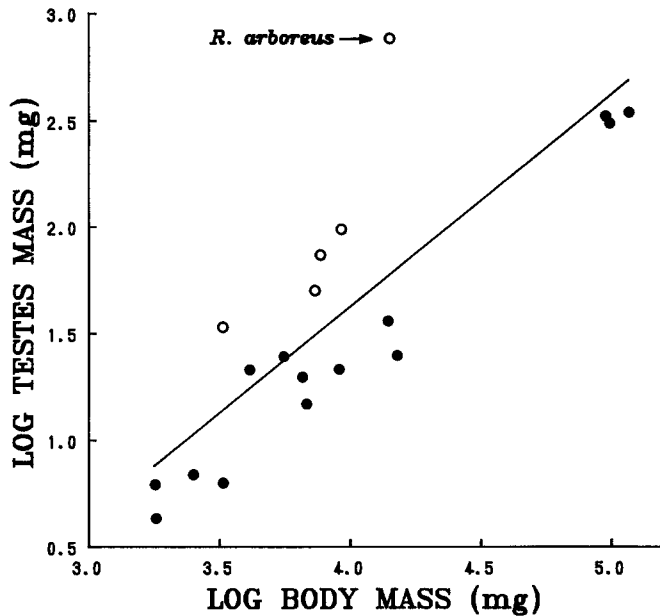
Fig. 1. Allometric relationship between body mass and testes mass (combined mass of both testes) in 19 anuran species. The *solid line* is the linear regression calculated from the log-transformed data of all species listed in Table 1: $Y = 1.00X - 2.36$ ($r^2 = 0.69$). o foam-nest building rhacophorids; ● other species
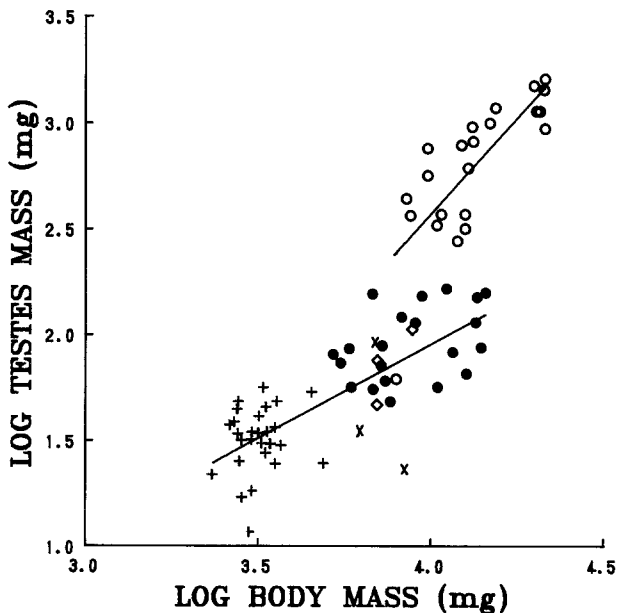

Fig. 2. Allometric relationship between body mass and testes mass (combined mass of both testes) in five species of foam-nest building rhacophorids. Each *symbol* indicates an individual male. The regressions are: $Y = 1.81X - 4.65$ for*R. arboreus* ($r^2 = 0.60$, $N = 22$) and $Y = 0.88X - 1.57$ for all other species ($r^2 = 0.58$, $N = 54$). o *R. arboreus;* + *R. schlegelii;* ● *R. viridis;* ◇ *R. owstoni;* × *P. leucomystax*

Testes size of foam-nest building rhacophorids

As mentioned before, foam-nest building rhacophorids are shown to have large testes relative to body size. For the five species, testes mass of an individual male is plotted against body mass on a logarithmic basis (Fig. 2). From the results, we can divide these rhacophorid spe-

cies into two species groups. One includes only *R. arboreus* and the other includes the four remaining species: *R. schlegelii*, *R. viridis*, *R. owstoni*, and *Polypedates leucomystax*. Analysis of covariance (Sokal and Rohlf 1981) indicates that regressions do not differ significantly among the latter four species in slope ($F_{3,46} = 1.15$, $P > 0.3$) and elevation ($F_{3,49} = 3.51$, $0.05 > P > 0.01$) at a 1% significance level. Therefore, a single regression line is obtained from the pooled data of these four species. This regression, however, differs significantly from that of *R. arboreus* in slope ($F_{1,72} = 8.75$, $P < 0.01$). Figure 2 shows that all points, except for one, plotted for data from *R. arboreus* are scattered above those of the other four species. Almost all of individual *R. arboreus* males have larger testes than similar-sized males of other rhacophorid species.

Discussion

Testes size and multi-male breeding

This study shows a clear trend in testes size among Japanese anurans. Since in this study the number of males sampled for each species varied greatly from 2 to 27 (Table 1), variable sample sizes may introduce some kinds of biases in analyzing the data. We may need data with more sufficient sample sizes to analyze the interspecific variation of testes size. We think, however, that the trend observed in this study is not very biased by the variable samples sizes, since standard deviations in the proportion of testes mass to body mass are relatively small in species that do not construct foam nests irrespective of sample size (Table 1), and the difference in the mean proportion of testes mass is large enough between the foam-nest building species and the other species.

In Japanese anurans, multi-male breeding has been reported for only two species of foam-nest building rhacophorids, *R. arboreus* (Kato 1956; Kasuya et al. 1987; Toda 1989) and *R. schlegelii* (Kikuchi 1988; Fukuyama 1991). Although we have no evidence at present, we think that multi-male breeding also occurs at relatively high frequency in other foam-nest building rhacophorids compared to species that do no construct foam nests. Since nest construction is a time-consuming event, females require 2 to 4 h to deposit eggs (Kato 1956; Kasuya et al. 1987; Toda 1989). In addition, since these rhacophorids are all prolonged breeders (see Maeda and Matsui 1989), the nightly sex ratio of breeding adults is highly skewed towards males, for example, ten males to one female in *R. arboreus* (Toda 1989). Long spawning duration and an unbalanced operational sex ratio enable several males to gather around a spawning pair, and this may lead to multi-male breeding. Relatively large testes size in foam-nest building rhacophorids may be related to the frequent occurrence of multi-male breeding. Large testes may have evolved in these multimale species through sperm competition.

There also exists another possibility that large testes are directly related to foam-nest construction; that is,

by releasing some substance from their testes, males may contribute to construction of the foam nest as well as females (see Duellman and Trueb 1986). However, females of *Chiromantis* have been observed to construct a foam nest in the absence of males (Coe 1974). Also, in *R. arboreus*, males participating in the spawning aggregation do not increase the size of foam nest; the nest size is not correlated with body size of males in amplexus or with the number of males (Kasuya et al. 1987). Thus, males probably contribute little to construction of the foam nest. In addition, the remarkably large testes in *R. arboreus* cannot be explained by the latter hypothesis since other foam-nest building rhacophorids also construct similar foam nests in a way similar to that of *R. arboreus*. On the other hand, the sperm competition hypothesis would successfully account for this phenomenon, as discussed below.

Ecological significance of large testes size in R. arboreus

The sperm competition hypothesis predicts that competition among sperm for fertilizing the deposited eggs is more intense in *R. arboreus* than in other foam-nest building rhacophorids. We examined this prediction by comparing the frequency of multi-male breeding in the field and the number of males participating in a spawning aggregation between *R. arboreus* and other foam-nest building species. Although quantitative data on this aspect of breeding ecology is very scarce for Japanese rhacophorid species, it is now available for *R. arboreus* (Toda 1989) and *R. schlegelii* (Fukuyama 1991). Since *R. arboreus* constructs foam nests chiefly on tree branches hanging above ponds, spawning behaviors are relatively easy to trace. Toda (1989) reported that 81.4% of the spawning pairs observed ($N=97$) included 1 or more additional males, and that the mean number of males participating in the spawning was 3.4 (SD = 1.9), the maximum number reaching 12. On the other hand, *R. schlegelii* constructs foam nests in underground holes in banks of still water. Therefore, it is very difficult to trace the pair formation and spawning process of this species. Fukuyama (1991) traced 9 radio-tagged gravid females in the field and found that 4 of the females were grasped by 2 or more males during spawning; the number of males participating in the spawning averaged 1.9 (SD = 1.3), and the maximum number was only 4. These results indicate that multi-male breeding occurs more frequently (Fisher's exact test, $P=0.022$), and more males participate in spawning by a single female ($t=2.29$, $df=104$, $0.05>P>0.01$) in *R. arboreus* than in *R. schlegelii*. Thus, intense sperm competition is suggested to occur in *R. arboreus*, supporting the sperm competition hypothesis concerning the evolution of testes size in Japanese anurans.

What are the ecological factors causing higher frequency of multi-male breeding and larger number of male participants in the spawning aggregations in *R. arboreus* than in *R. schlegelii*? We think that arboreal breeding is one of the important factors. In the species constructing foam nests within the soil, such as *R. schlegelii*, it is relatively difficult for single males to locate amplexed pairs constructing foam nests. In addition, since the underground holes where nests are formed are narrow, additional males participating in the spawning are greatly limited in number. An amplexed pair of *R. arboreus* constructs a foam nest chiefly in an open space such as on a tree branch, which is easily detected and approached by other males. Such breeding habits, together with the long duration of spawning, increase the probability that additional males gather around the pair and participate in spawning.

We have documented that foam-nest building rhacophorid species, especially *R. arboreus*, have large testes relative to body size as compared to other anuran species in Japan. The close relationship between relative testes size and frequency and intensity of multi-male breeding strongly suggests the importance of sperm competition in the evolution of relative testes size. At present, however, we have little quantitative data on the breeding systems of Japanese anurans, especially of rhacophorid species inhabiting the Ryuku Islands, such as *R. viridis*, *R. owstoni*, and *P. leucomystax*. We need more detailed comparisons of relative testes size and reproductive ecology among foam-nest building rhacophorids with relatively large testes.

Testes size and sperm depletion effect

In the present paper, we have tried to explain the evolution of relative testes size in Japanese anurans by the sperm competition hypothesis. However, the trend observed in this study may also be explained by another hypothesis: sperm depletion hypothesis (Cartar 1985). In addition to sperm competition, seasonality of breeding is considered to be important for the evolution of relative testes size in mammals (Harcourt et al. 1981; Harvey and Harcourt 1984; Kenagy and Trombulak 1986). In Japanese anurans, the duration of breeding season varied from 1 week to almost 1 year from species to species, but we cannot find any clear relationship between the duration of breeding season and relative testes size. Foam-nest building rhacophorids with large testes are all prolonged breeders; *R. arboreus* breeds for 1 to 3 months (Kato 1956; Toda 1989). Many other species with small testes also breed for a longer period. For example, *B. buergeri* breeds for about 3 months (Fukuyama and Kusano, unpublished), *R. rugosa* for 4 months (Okochi 1979), *M. ornata* for 5 months, *B. japonica* for about half a year, and *B. marinus* probably all year round (Maeda and Matsui 1989). Seasonality of breeding is not considered to be important for the variation of relative testes size in Japanese anurans.

The mean number of matings for males during a breeding season is expected to be higher in foam-nest building rhacophorid species than in other species since multi-male breeding occurs frequently in the former species. Such high mating frequency may affect the evolution of large testes size in foam-nest building rhacophorids (Iwasawa 1954; Cartar 1985). At present, however,

we do not have any evidence that males participate in spawning more frequently in species with a multi-male breeding system than in other species. Since the sex ratio of a breeding population is skewed toward males in foam-nest building rhacophorids, the mean number of matings by males may not be as large as we expect. For example, the sex ratio (male/female) is estimated to be at least more than 2, and the average number of male participants during female spawning is 3.4 in *R. arboreus* (Toda 1989). Therefore, the mean number of matings can be estimated to be less than 1.7 for males. This value may not be large enough to explain the huge testes size in *R. arboreus* only by sperm depletion effect.

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