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Male Reproductive Cycle of the Toad *Bufo melanostictus* in Taiwan

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ABSTRACT—The male reproductive cycle of the toad, *Bufo melanostictus*, was studied with emphasis on spermatogenic activity, plasma androgen, and changes in the weights of testes, liver, and fat bodies. A total of 98 toads were collected between March 1990 and March 1991 in central Taiwan. Histological evidence indicated that the spermatogenic cycle of this toad is of a fluctuating continuous type. Although cell nests of all spermatogenic types were present every month of the year, the greatest intensity of spermatogenic activity, as expressed by the presence of sperm bundles, occurred in March. Both testicular weight and plasma androgen levels peaked in March. The weights of fat bodies peaked in July, which was not coincident with the beginning of breeding. Combined data from spermatogenic activity, plasma androgen levels, and changes in the weights of testes, fat bodies, and livers revealed that *B. melanostictus* is a continuous breeder. However, its annual reproductive cycle could be divided into 4 periods: 1) breeding period (February-April); 2) post-breeding period (May-June); 3) reproductive energy preservation period (July-September); and 4) torpid period (October-January).

INTRODUCTION

Annual spermatogenic cycles in anurans have been grouped into 3 categories: discontinuous, potentially continuous, and continuous types (Lofts, 1974). The discontinuous type, commonly found in temperate-zone species such as *Rana esculenta* (Lofts, 1964) and *R. temporaria* (Lofts *et al.*, 1972), generally has discrete seasonal cycles of reproduction with pronounced changes in gonad size, gamete production, and sex accessory structure. The potentially continuous type exhibits a partial cessation of spermatogenic activity during some seasons of the year but primary spermatogonia remains sensitive to gonadotrophic stimulation. *Hoplobatrachus tigrinus* (previously known as *Rana tigrina*) is an example of this type (Saidapur and Nadkarni, 1975). Species inhabiting tropical regions, where climatic conditions do not show appreciable fluctuation, have developed the continuous type of spermatogenetic cycle, and *R. cyanophlyctis* (Saidapur and Nadkarni, 1973) and *Bufo melanostictus* (Kanamadi *et al.*, 1983; Saidapur, 1983) exhibit this type.

Studies of plasma hormone levels in wild animal species provide useful information on endocrine control mechanisms. Characterizations of the annual cycle of plasma sex steroid levels are especially important in the study of reproduction in species with a definite breeding season. Seasonal changes in plasma steroid levels have been reported in a number of anuran species: *R. esculenta* (d'Istria *et al.*, 1974), *R. pipiens* (Basu and Nandi, 1965; Wada *et al.*, 1976), *B. mauritanicus* (Siboulet, 1981), *R. catesbeiana* (Licht *et al.*, 1983; Yoneyama and Iwasawa, 1985), *Dicroglossus occipitalis* (kuhn *et al.*, 1987), *R. nigromaculata* (Tanaka *et al.*, 1988), *R. perezi* (Delgado *et al.*, 1989), *B. japonicus* (Itoh and Ishii, 1990a, b), *R. rugulosa* (Kao *et al.*, 1993), and *B. bankorensis* (Huang *et al.*, 1996).

We have investigated the male reproductive cycle of the toad, *B. melanostictus*, in central Taiwan. This paper reports: 1) the circa-annual changes in the weights of body, testis, liver, and fat body; 2) the spermatogenic cycles; 3) plasma androgen levels; and 4) the correlations of plasma androgen levels with environmental factors (rainfall and temperature), spermatogenesis and other reproductive activities.

MATERIALS AND METHODS

Study site

The toads were collected from 5 sites along the Honken River in Deh-Ken (24° 10'N, 120° 43'E), Taichung City, central Taiwan. Data for temperature and rainfall for 1990–1991 in central Taiwan are summarized in Fig. 1 (Data are from the Shuijaitou Weather Station, Central Weather Bureau, ROC). According the wild observations, such as mating call, amplexus behavior, tadpole emergent time, and collection number of toads, four periods in an annual cycle, February to April, May to June, July to September, and October to January, were determined in the present study.

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Collection of blood, tissue and organs

Toads were collected at night from the 5 sites on the 5th, 15th, and 25th days of each month from March 1990 through March 1991. The numbers of toads collected each month are shown in Table 1. In the torpid period, it was difficult to find toads, especially in July. The collections of blood, tissue and organs were done in the laboratory on the next day following capture of the toads. All toads were weighed and measured before dissection; the fresh weights of testes, fat bodies, and liver were recorded. To minimize the body weight differences of individual toads, weights of organs were also expressed as an organosomatic index as (gram organ weight/gram body weight) × 100%.

Blood was collected in heparinized microtubes from the conus arteriosus after the toads were anesthesized with diethyl ether. The blood sampling was usually completed within 10 min for each individual. The blood was centrifuged at $10^3 \times g$ for 10 min, and plasma (0.5 to 1.2 ml) was collected and then stored at -20°C unti the assay of androgen.

Histological examination

The right testis of each animal was fixed in Bouin's solution, embedded in paraffin, serially sectioned at 6 μm, and stained with hematoxylin and eosin. Spermatogenetic activity was assessed as described by Saidapur (1983): Stage 0, primary spermatogonia; stage I, secondary spermatogonia; stage II, primary spermatocytes; stage III, secondary spermatocytes; stage IV, presence of sperm bundles in the seminiferous tubule; and stage V, appearence of mature sperm in the seminiferous tubule lumen. The intensity of spermatogenetic activity is expressed by the number of sperm bundles in the seminiferous tubules. Sperm bundles were coounted from 20 random sections of each testis. The number of sperm in the seminiferous tubule was calculated as the area of free sperm in the seminiferous tubule lumen divided by the total area of seminiferous tubule lumen; values are expressed as abundant (> 50%), median (30%-50%) and few (< 30%).

Radioimmunoassay of androgen levels in plasma

Plasma androgen was measured by a radioimmunoassay described previously by Kao *et al.* (1993). Androgens in the samples were extracted with diethyl ether; the average recovery of the added testosterone was 75.3 ± 4.3%. The cross-reactivites of the antiserum with steroids were tested for testosterone (100%), dihydrotestosterone (74%), androstenedione (1.23%), and androstenediol (0.59%). Thus, the data are expressed as "androgen" which mainly represents testosterone and dihydrotestosterone present in the plasma. Intra- and inter-assay variabilities had been previously established at 6.7% and 12.9%, respectively.

Statistical analysis

Analysis of variance (ANOVA) and Duncan's multiple range tests were used to examine differences among the numerical mean values. Pearson's correlations analyses were performed for all variables (SAS, 1994). Regression analysis was performed between SB, FBSI, LSI, as well as TW (for the explanations of abbreviations, see Table 4), and environmental variables. In the analyses, SB, FBSI, LSI, AL, and TW were treated as dependent variables, whereas the climatic factors of monthly air temperature and rainfall were treated as independent variables. A probability value of 0.05 or less was considered to indicate significance.

RESULTS

Observations of reproductive cycle, and climatic factors

The mating call and amplexus were observed in February and tadpoles appeared at the end of March. The breeding season continued through May; the numbers of adults and tadpoles decreased after the end of May and the tadpoles disappeared in June. In October-December, when temperatures decreased to 17°C, it was difficult to find and collect toads. As shown in Fig. 1, the highest mean temperatures were in July 1990, at 28°C, with a decrease to the lowest at 17°C in January 1991. The period between April and August 1990 was the rainy season.

Histological observations of testes

The histological examinations of *B. melanostictus* testes collected monthly from March 1990 through March 1991 revealed that the spermatogenetic activities in the months of

Table 1. Changes in the mean weights of the body, testis, liver, and fat body during an annual reproductive cycle of male *Bufo melanostictus*

Month	N*	Body		Organ weights		
		length (cm)	weight (g)	testis (mg)	liver (g)	fat bodies (g)
1990						
Mar	13	64.0 ± 3.2	25.6 ± 1.4	62.2 ± 3.3	0.61 ± 0.01	0.12 ± 0.00
Apr	14	61.7 ± 3.0	25.1 ± 1.7	44.2 ± 1.2	0.91 ± 0.05	0.52 ± 0.03
May	10	64.0 ± 3.7	27.2 ± 2.8	35.5 ± 3.4	0.93 ± 0.03	0.74 ± 0.04
Jun	11	63.3 ± 4.0	25.3 ± 1.4	51.0 ± 4.3	0.80 ± 0.05	0.83 ± 0.05
Jul	3	63.4 ± 7.2	26.6 ± 1.7	40.0 ± 2.2	0.94 ± 0.06	4.00 ± 0.07
Aug	3	61.0 ± 4.4	20.4 ± 2.7	33.3 ± 1.2	0.60 ± 0.03	0.23 ± 0.00
Sep	8	57.1 ± 4.1	19.1 ± 1.0	20.0 ± 1.6	0.57 ± 0.04	0.12 ± 0.01
Oct	2	51.0	16.8	23.6	0.43	0.15
Nov	2	57.0	17.1	30.0	0.54	0.10
Dec	1	59.0	20.9	30.0	0.70	0.00
1991						
Jan	11	56.8 ± 3.5	16.9 ± 1.6	34.4 ± 2.2	0.41 ± 0.03	0.00 ± 0.00
Feb	10	67.0 ± 6.6	26.9 ± 1.8	50.0 ± 4.7	0.60 ± 0.06	0.10 ± 0.00
Mar	10	64.0 ± 3.2	25.3 ± 1.4	61.3 ± 5.7	0.60 ± 0.03	0.14 ± 0.00

Data are expressed as the means ± SE. N* indicates the monthly sample sizes.

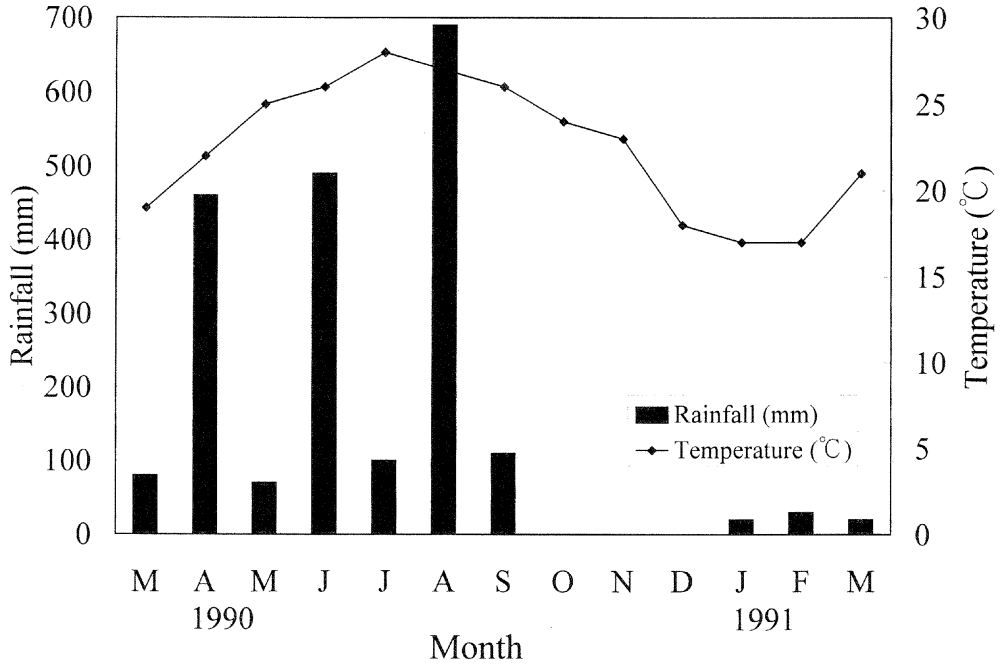

Fig. 1. Monthly changes in rainfall and mean air temperature in Taichung, Taiwan.

Table 2. Most advanced stage of spermatogenesis found in testis of *Bufo melanostictus* in different months of the year

	1990										1991		
SS[a]	M	A	M	J	J	A	S	O	N	D	J	F	M
IV[b]		20%	80%		40%		40%						
V	100%	80%	20%	100%	60%	100%	60%	100%	100%	100%	100%	100%	100%

[a] SS denotes the stage of spermatogenesis.
[b] stages IV and V represent the status of the seminiferous tubules, containing sperm bundles and mature sperm, respectively.

Table 3. Monthly change of spermatogenetic intensity: number of sperm bundles (SB, mean ± SE) and number of sperm in seminiferous tubules (SN) of *Bufo melanostictus*

	1990										1991		
	M	A	M	J	J	A	S	O	N	D	J	F	M
SB	7.6 ± 1.0	3.2 ± 0.5	3.2 ± 0.6	7.3 ± 0.8	6.0 ± 1.1	6.3 ± 0.9	4.2 ± 1.0	5.0	3.5	1.0	4.8 ± 0.6	4.8 ± 0.4	8.4 ± 1.0
SN	A	F	F	F	F	F	F	F	F	F	F	M	A

A, abundant (> 50%); M, median (30%–50%); F, few (< 30%)

January-March, June, August, and October-December were at stage V; while in April, May, July, and September the activities were at both stage IV and stage V (Table 2).

The number of sperm bundles increased from June 1990, then declined to the lowest in December, and peaked in March 1991 (Table 3). In the 4 annual periods (February to April, May to June, July to September, and October to January), the mean numbers of sperm bundles were 6.3, 5.2, 5.5, and 3.6, respectively. No significant difference was found among these 4 periods ($F_{3,9} = 1.01$, $p = 0.43$), suggesting that spermatogenic intensity does not vary significantly throughout the year. The numbers of sperm bundles were significantly correlated with LSI ($r = 0.56$, $p < 0.05$) and plasma androgen ($r = 0.82$, $p < 0.05$) (Table 4).

Annual changes in TW, LSI, and FBSI

As indicated in Table 1, the mean testis weight (TW) showed significant variations between months ($F_{1,9} = 5.92$, $p < 0.05$) and had significant correlations with both sperm bundles ($r = 0.65$, $p < 0.05$) and plasma androgen ($r = 0.78$, $p < 0.01$) (Table 4). The mean liver somatic indices (LSIs) were 2.4%, 3.1%, 2.8%, and 3.1% (Fig. 2A) for the periods February-

Table 4. Correlation coefficients (r) of sperm bundles (SB), fat body somatic index (FBSI), liver somatic index (LSI), level of plasma androgen (AL), testis weight (TW), temperature (TEM), and rainfall (RF) of male *Bufo melanostictus* during an annual reproductive cycle

	SB	FBSI	LSI	AL	TW	TEM	RF
SB		0.15	−0.56*	0.62*	0.65*	0.31	0.23
FBSI			0.32	−0.19	0.07	0.52	0.07
LSI				−0.53	−0.53	0.29	0.31
AL					0.78**	0.16	0.36
TW						0.20	0.06

Correlation coefficients (r) were analyzed from the monthly means of these parameters. *, $0.01 < p < 0.05$; **, $p < 0.01$.

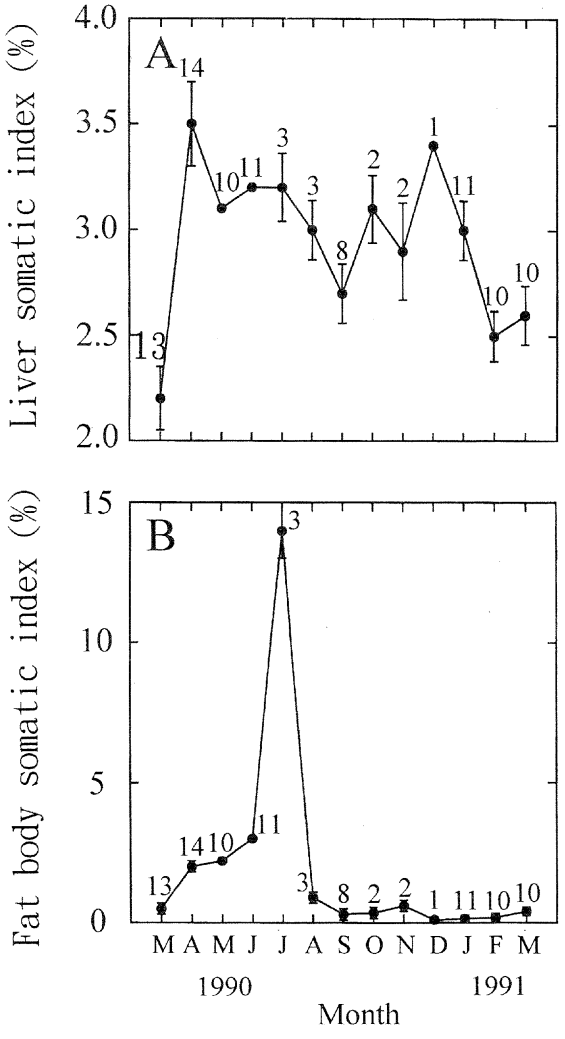

Fig. 2. Monthly changes of the lvier somatic index (**A**), and fat body somatic index (**B**). The data are expressed as mean ± SE, and the numbers above the dots indicate monthly sample sizes.

April, May-June, July-September, and October-January, respectively, and showed no significant difference ($F_{3,9} = 0.99$, $p = 0.44$). The mean fat body somatic indices (FBSIs) were 0.3%, 0.5%, 3.1%, and 5.1% (Fig. 2B) for the periods October-January, February-April, May-June, and July-September, respectively, and also showed no significant variations among

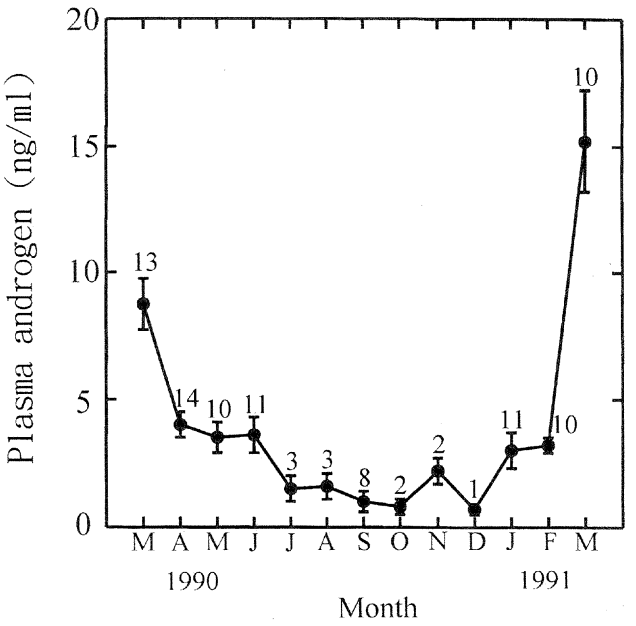

Fig. 3. Monthly changes of plasma androgen levels during an annual reproductive cycle of *Bufo melanostictus*.

the 4 periods ($F_{3,9} = 1.18$, $p = 0.37$).

Annual cycle of plasma androgen levels

Plasma androgen levels peaked in March (mean value=12.0 ng/ml), and then decreased to the lowest in December (0.5 ng/ml) (Fig. 3). A significant periodic trend in plasma androgen levels was recorded ($F_{3,9} = 3.96$, $p < 0.05$).

The correlations among sperm bundles (SB), LSI, FBSI, levels of plasma androgen (AL), TW, temperature, and rainfall are summarized in Table 4. As indicated, AL was positively correlated with SB and TW.

DISCUSSION

Reproductive cycle

The reproductive cycle of *B. melanostictus* may be divided into 4 periods: breeding, post-breeding, reproductive energy preservation and torpid periods. The numbers of sperm bundles in the seminiferous tubules, the numbers of free sperm, and plasma androgen levels in February-April were all higher than during the other 3 periods. Field observations showed that the breeding behaviors occurred in February-April. Thus, February-April is considered to be the breeding period. In May-June, the numbers of sperm bundles, free sperm, and adult toads were lower, and this is considered to be the post-breeding period. In July-September, the numbers of sperm bundles and free sperm were low; the FBSI value in this period was indeed higher than in the other three periods. July-September was thus considered the reproductive energy preservation period. In October-January, the numbers of sperm bundles and free sperm were lowest; at the same time, adult toads were difficult to find in the field. This period was thus

considered the torpid period.

Testicular activity

Amphibians inhabiting tropical areas, where climatic conditions do not show appreciable fluctuations, have often developed a continuous type of spermatogenic cycle; whereas other species in temperature regions have developed a discontinuous or potentially continuous type of spermatogenic cycle (Basu and Nandi, 1965; Lofts, 1974). In species with a continuous type of spermatogenic cycle, the spermatozoa are generally produced throughout the year and the testes always contain spermatic cell nests as well as a complete spectrum of spermatogenic stages (Sun, 1979; Saidapur and Kanamadi, 1982; Saidapur, 1983). In contrast, the spermatogenic activity of species living in temperature regions in restricted to late spring and summer; such discontinuous production of spermatozoa has been attributed, at least in part, to a seasonal lowering of environmental temperatures and an accompanying decline of gonadtropin output (Lofts, 1974). Mondal and Basu (1960), Lofts (1974), and Saidapur (1983) proposed that the spermatogenic cycle of species which have free sperm in the seminiferous tubules belongs to the continuous type. In other words, the classification of spermatogenic cycles of anuran species belonging to continuous or discontinuous types depends on the presence or absence of free sperm in the seminiferous tubules. As observed in the present study, *B. melanostictus* had free sperm in the seminiferous tubules the entire year. According to the categorizations by Lofts (1974) and Saidapur (1983), the spermatogenic cycle of *B. melanostictus* thus belongs to the continuous type. However, a comparison of the numbers of free sperm showed that they were significantly higher in the breeding period than during any of the other 3 periods (Table 3). Such observations reveal that although *B. melanostictus* belongs to the continuous type of spermatogenic cycle, it has a distinct breeding period. Accordingly, it is inappropriate to determine the nature of the reproductive cycle of toads by depending solely on the presence of free sperm in the seminiferous tubules. We thus propose that the continuous type of spermatogenic cycle of anurans may be divided into the constantly continuous type (e.g., *R. cyanophlyctis*; Saidapur and Kanamadi, 1982), and the fluctuating continuous type (e.g., *B. melanostictus*, present study).

Changes in the weights of the testes have also been used as a basis to determine the reproductive cycle of amphibians (Lofts *et al.*, 1972; Rastogi *et al.*, 1986). The results of the present study reveal that the annual variations of testicular weight of *B. melanostictus* show a significant difference between months, and that testicular weight was significantly correlated with the number of sperm bundles and plasma androgen levels (Table 3). Thus, seasonal variations in testicular activity are generally reflected in gonadal weight. Such a pattern is generally similar to that observed in *B. bankorensis*, a species exhibiting a potentially continuous type of spermatogenic cycle (Huang *et al.*, 1996), and to that of species with a discontinuous-type testicular cycle, such as *R. temporaria*, in which testicular weight is an appropriate index of testicular function (Lofts *et al.*, 1972).

Androgen patterns and correlation with reproductive cycle

The patterns of seasonal plasma androgen levels of *B. melanostictus* observed in the present study are very similar to those previously reported *Dicroglossus occipitalis* (Kuhn *et al.*, 1987), *B. japonicus* (Itoh and Ishii, 1990a), *Rana rugulosa* (Kao *et al.*, 1993) and *B. bankorensis* (Huang *et al.*, 1996); all of these anurans exhibited a distinct circulating androgen peak during their breeding period. It is usually accepted that androgen is related to the initiation of breeding activity and the expression of sexual behavior (Duellman and Trueb, 1986). Although the exact functions of androgen in *B. melanostictus* are unknown at present, the finding that plasma androgen levels are maximal in the breeding period suggtests that the mating behavior is also probably androgen-dependent in this species. Siboulet (1981) also demonstrated that circulating androgen levels in male *B. mauritanicus* are higher during periods of amplexus.

In some of the anurans investigated, a notable decrease in plasma androgen levels and an increase in seprmatogenic activity were observed after the breeding period [e.g., *B. mauritanicus* (Siboulet, 1981) and *Pachymedusa dacnicolor* (Rastogi *et al.*, 1986)]. A possible androgen negative feedback mechanism at the hypothalamus-hypophysis axis level has been proposed (Rastogi *et al.*, 1976; Delgado *et al.*, 1989; Itoh and Ishii, 1990a). It is generally believed that in early phases of the spermatogenic cycle of anurans, the mitotic proliferation of primary and secondary spermatogonia is gonadtropin-independent (Lofts, 1974), and that the meitotic proliferation of secondary spermatocytes, formation of sperm bundles, and presence of free sperm are androgen-dependent (Saidapur, 1983). The present study of *B. melanostictus* revealed that the higher levels of androgen during the breeding period from February to April were observed coincident with the increase of sperm bundles and free sperm in the seminiferous tubules. Such observations are consistent with those reported for other anurans (Rastogi *et al.*, 1976; Kobayashi and Iwasawa, 1986; Saidapur, 1983; Kao *et al.*, 1993; Huang *et al.*, 1996).

Relations of changes of fat body and liver weights to the reproductive cycle

Lipids are the most efficient storage form of the 3 potential energy compounds (lipids, glucose, and proteins) in anurans (Long, 1987). The patterns of annual lipid variation have been observed in many anurans; body lipid reduction may reflect its metabolic use during dormancy, for vitellogenesis, and possibly for steroid production during the reproductive period (Chieffi *et al.*, 1975; Rastogi *et al.*, 1978; Morton, 1981; Pramoda and Saidapur, 1984; Kanamadi and Saidapur, 1988). As observed in the present study, no significant correlations were observed between FBSI and various reproductive parameters (Table 4); the role of fat bodies during the annual reproductive cycle of *B. melanostictus* is still nuclear and

requires further investigation. Liver glycogen also serves as a metabolic substrate in anurans (Brenner, 1969). The negative correlations between LSI and sperm bundles observed in the present study suggest that the energy accumulation in the liver may be used in the spermiogenesis activity of *B. melanostictus*.

Association of environmental factors with the reproductive cycle

Three major environmental factors have been implicated in the regulation of the amphibian breeding cycle: rainfall, photoperiod, and temperature (Lofts, 1974). Rainfall has been shown to influence the breeding behavior in various anurans (Lofts, 1974). It was demonstrated that photoperiod potentially modifies testicular activity in *R. esculenta* (Rastogi *et al.*, 1976). Temperature, in contrast, has been shown to play an important role in regulating plasma androgen levels and in synchronizing the different phases of the seasonal testicular cycle in several anurans (Rastogi *et al.*, 1976, 1978; Iela *et al.*, 1980; Pancak and Taylor, 1983; Pierantoni *et al.*, 1985). It was further affirmed that temperature was the most important factor and that photoperiod plyaed only a permissive role in the mediation of temperature influence on the gametogenic and endocrine activity of *R. esculenta* from the temperate region (Rastogi *et al.*, 1978). Our present study of the subtropical anuran species, *B. melanostictus*, revealed that no apparent correlations existed between temperature and testicular activities or between rainfall and testicular activities. The relation of photoperiod to testicular activities of the toad was not investigated in this study.

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