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Research article

The male reproductive pattern of the Isan big-headed frog (*Limnonectes isanensis*, McLeod, Kelly, and Barley, 2012) at the Loei river in Loei province, Thailand

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Abstract

Importance of the work: Increasing research on the reproductive patterns of species threatened by capture for food leads to enhanced conservation data.

Objectives: To monitored reproductive pattern of the male Isan big-headed frog, *Limnonectes isanensis*.

Materials and Methods: Samples were collected monthly at the Loei river in Phu Luang district, Loei province, over a period of one year. The testes were excised for examination of annual morphological and histological changes, and blood was withdrawn via cardiac puncture for the determination of testosterone levels by radioimmunoassay (RIA).

Results: The testes regressed from March to May, recrudesced from June to August and hypertrophied from September to February. Histological analysis of the seminiferous lobules exhibited that from May to August no luminal sperm bundles or free sperm were present, but abundant cystic spermatocytes were observed; from September to February, abundant sperm bundles and free sperm were observed, and fewer free sperm were present from March to April. The testosterone levels of male *L. isanensis* were low from March to August, gradually increased in September and October, and surged markedly from November until February of the following year.

Main finding: The combined data on annual alterations in testicular morphology, spermatogenetic activity, and plasma testosterone levels demonstrate that the reproductive pattern of male *L. isanensis* follows an associated reproductive pattern.

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Introduction

The Isan big-headed frog, *Limnonectes isanensis*, belongs to the order Anura of the family Ranidae. Although it was recently described as a new species by McLeod et al. (2012), it was previously classified *as L. megastomias*, a species from northeastern Thailand that belongs to the *L. kuhlii* complex (McLeod, 2008). *L. isanensis* is exclusively distributed in evergreen forests along five- to ten-meters-wide hillside streams that feed the Loei River. Most individuals are found two to three meters from streams on rocks along the stream bank, amidst leaf litter and/or at streamside pools. A few published reports on the genus *Limnonectes* are available. Its habitat occurs at high elevations, and the population of the *L. kuhlii* complex in Thailand is distributed from north to south along the mountainous western edge of the kingdom, in the mountainous north and in the northeastern region along the Korat Plateau. To date, there is no published information on the reproductive pattern of male *L. isanensis*.

No generalized male reproductive pattern is valid for all anuran species (Vitt and Caldwell, 2009). Amphibian reproductive patterns are classified into three types, discontinuous, potentially continuous, and continuous, according to the changes in the annual gonadal cycle (Lofts, 1974). The majority of anuran species inhabiting the temperate zone has a discontinuous type of reproductive pattern in which spermatogenesis is impaired or completely interrupted during part of the year (Vitt and Caldwell, 2009), for example, *Rana temporalia* (Van Oordt, 1960), *Pleurodema bufonina* (Cei, 1980), *Pachymedusa dacnicolor* (Rastogi et al., 1986), *Bufo japonicus* (Itoh et al., 1990), *R. dalmatina* (Guarino and Bellini, 1993) and *R. italica* (Guarino et al., 1993). Most anurans inhabiting the tropical zone have a reproductive pattern of the continuous type in which the different stages of spermatogenesis occur throughout the year (Vitt and Caldwell, 2009); these include, for example, *R. catesbeiana* (Yoneyama and Iwasawa, 1985), *Polypedates maculatus* (Kanamadi and Jirankali, 1992), *R. rugulosa* (Kao et al., 1993), *Leptodactylus ocellatus* (Vivas et al., 1995) and *Amolops larutensis* (Emerson and Hess, 1996). In the potentially continuous type, there is partial suppression of spermatogenesis during some months of the year, but the primary spermatogonia remain sensitive to gonadotrophic stimulation (Saidapur and Nadkarni, 1975); this occurs, for example, in *R. trigina* (Saidapur and Nadkarni, 1975) and *Bufo bankorensis* (Huang et al., 1996).

The reproductive patterns of many amphibian species depend not only on changes in testicular structure and spermatogenic activity but also on annual variations in plasma testosterone levels (Rastogi and Iela, 1992). The annual change in plasma testosterone levels depends on whether the species displays a dissociated or an associated reproductive strategy (Shalan et al., 2004). The dissociated reproductive strategy is characterized by high plasma testosterone levels coincident with inactive spermatogenesis (Delgado et al., 1989), whereas in the associated reproductive strategy, high plasma testosterone levels change simultaneously with active spermatogenesis (Rastogi et al., 1986).

The objective of this study was to categorize the male reproductive pattern of *L. isanensis* by monitoring annual changes in testicular structure, spermatogenic activity, and plasma testosterone levels, with the aim of determining whether the species exhibits an associated or dissociated reproductive pattern.

Materials and Methods

Animal samples and sample site

Male adult *L. isanensis* were employed in this study. Identification of adult males and females is based on their external morphology; the head of the male is larger than that of the female (Figs. 1A and 1B). We collected monthly ten adult males from the Loei river, Loei Wang Sai subdistrict, Phu Luang district, Loei province, in the northeastern part of Thailand from June 2012 until May 2013. The specimens were collected along hillside streams five to ten meters wide on Phu Luang Mountain at an elevation of five hundred meters above sea level (17°03’40.0”N 101°31’09.3”E) within the evergreen forest. The populations are widely distributed approximately two to three meters from streams on rocks along the stream margins, underlying leaf litter and in streamside pools. The annual changes in rainfall, relative humidity, and air temperature at the Loei river, Phu Luang district, Loei province are summarized in Fig. 2. We captured individual frogs, placed them in plastic bottles, and transported them to the Physiology Laboratory Division at the Department of Zoology, Faculty of Science, Kasetsart University. Each frog was lightly anesthetized with diethyl ether and weighed before dissection to determine the internal reproductive morphology and testicular histology and to obtain blood for testosterone determination.

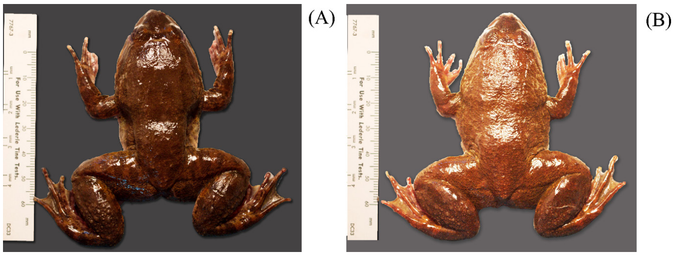

Fig. 1 (A) the male *L. isanensis*; and (B) the female *L. isanensis*

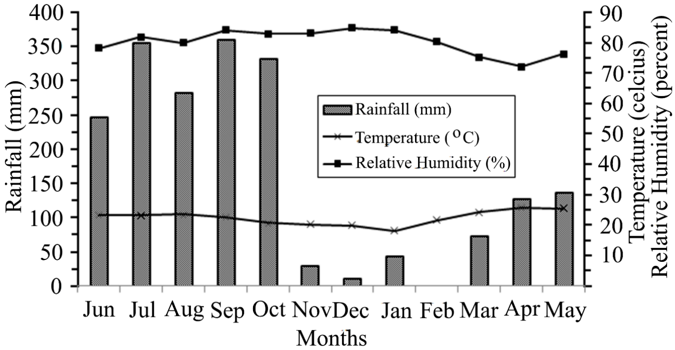

Fig. 2 Monthly changes in rainfall, relative humidity and air temperature in Phu Luang district, Loei province, northeastern part of Thailand

Internal reproductive morphology determination

Male frogs were sacrificed, and their internal testicular morphology was investigated to identify the regressed and hypertrophied testes, respectively.

Testicular histological analysis

The samples were excised via the abdomen, and the testes were collected for the determination of changes in the seminiferous lobule. The samples were fixed in 10% neutral buffered formalin for 24 hr, rinsed with tap water for one and a half hours and transferred to a bottle containing 15 mL of 70% ethanol. The samples were then processed by the paraffin technique. The tissue was cut in cross section to 6 µm thickness using an AO Rotary Microtome Model 820. The sections were stained with hematoxylin and eosin (Avwioro, 2011).

Radioimmunoassay of testosterone

Blood samples were collected via cardiac puncture and used to monitor annual changes in the testosterone profile. The samples were collected through a 29G x 1/2 needle attached to a 1-mL syringe containing heparin solution, transferred to 1.5-mL vials, and kept at 4ºC until centrifugation within 3–4 hours. The blood was centrifuged at 1,600 x g for ten minutes. The plasma was collected by aspiration and frozen at 79ºC for later analysis of plasma testosterone. The plasma levels of testosterone were measured by 125I radioimmunoassay (RIA). In the assay, a 500-µL sample of plasma was added to 5.0 mL of dichloromethane in a screw-top glass extraction tube. The tube was capped, and the extraction mixture was mixed for sixty minutes by gentle inversion on an end-over-end rotator; the sample was then centrifuged for five minutes at 1,500 x g to separate the layers. The upper phase was aspirated without disturbing the interface, and 2.0 mL of the lower phase was transferred to a clean 12-mm x 75-mm glass tube and evaporated to dryness under a stream of nitrogen at 37ºC. Finally, the sample was reconstituted with 200 µL of testosterone buffer. The testosterone radioimmunoassay was performed using the Coat-A-Count® Testosterone Kit (Diagnostic Products, Los Angeles, CA, USA). The intra-assay and inter-assay variations, expressed as coefficient of variation (%CV), were 8.4 and 7.9 percent, respectively. The approximate sensitivity of the assay was 10 pg/mL. The cross-reactivity with androstenedione was 0.5 percent. The spiking recovery values averaged 98.3±0.6 percent. The measurement results obtained for 50.0, 25.0, and 12.5 percent dilutions of a solution containing 7300 pg/µL testosterone were 3490, 1700, and 780 pg/µL, respectively.

Statistic analysis

Testosterone levels were expressed as mean ± standard deviations. Normal distribution and homogeneity of variance of the data were analyzed using the Shapiro-Wilk test and the Levene's test, respectively. Since the testosterone data were non-normal distribution, heteroscedasticity of variance, and no repeated measurement, the Kruskal-Wallis H test was used to test the mean rank differences in the testosterone levels over twelve months and subsequently, the Mann-Whitney U test as post hoc tests was employed to compare the mean rank differences in the male testosterone levels between every two months (month to month). The threshold of significance was set to $p < 0.05$ for the male testosterone with SPSS software (version 20; SPSS Inc.; Chicago, IL, USA).

Ethics statements

This study was carried out under Animals for Scientific Purposes Act, B.E. 2558 (A.D. 2015) (License no. U1-020037-2558).

Results

Annual changes in testicular morphology

The testes of *L. isanensis* are paired, oval-shaped organs lying on the ventral side of the kidneys. They are situated at the medial anterior part of the kidneys. In the dorsal part of a testis, the two sheets of peritoneum form a double-layered mesochrium that contains an efferent duct system, the vas deferens, that transports sperm to the kidney. The observed annual changes in testicular morphology in male *L. isanensis* were as follows: the testes were recrudesced from June to August, hypertrophied from September to February, and regressed from March to May (Fig. 3).

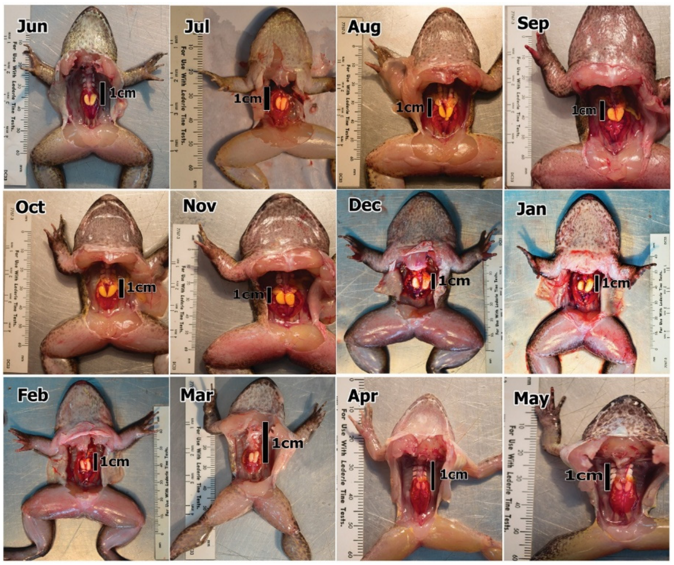

Fig. 3 The testicular morphological changes of *L. isanensis* from June to December (2012) and from January to May (2013)
Note: Jan, January; Feb, February; Mar, March; Apr; April, Jun; June, Jul; July; Aug, August; Sep, September; Oct, October; Nov, November and Dec, December

Annual alterations in the histology of seminiferous lobules

The testes of *L. isanensis* are occupied by masses of convoluted seminiferous lobules and interstitial cells known as Leydig's cells are located between the seminiferous lobules. Spermatogenesis occurs within additional compartments of the seminiferous lobules that are formed by a number of spherical vesicles called germinal cysts. The walls of the germinal cysts are composed of one or a few Sertoli cells. The seminiferous lobules were found to contain germinal types of spermatogonia and spermatocytes inside the cysts and abundant sperm bundles and free sperm in the lumen (Figs. 4A and 4B). From May until August, there were no free sperm in the lumen, but only germinal types of spermatogonia and spermatocytes were present in the germinal cysts. From September to February, masses of sperm bundles and free sperm were present in the lumen of the seminiferous lobules. During March and April, free sperm were present in the lumen of the seminiferous lobules in lower amounts, and most of the germinal cysts were in the spermatogonia and spermatocyte stages (Fig. 5).

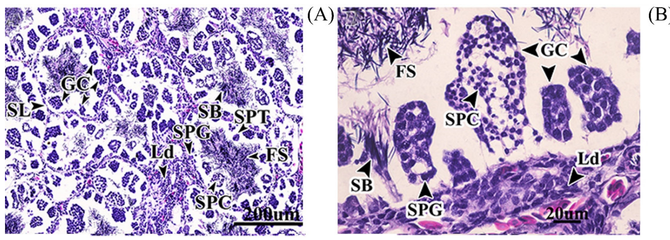

Fig. 4 (A) Photomicrograph of testicular architecture of male *L. isanensis*; and (B) High magnification of (A)
Note: GC, germinal cyst; SB, Sperm bundle; FS, Free sperm; Ld; Leydig's cell; and SL, Seminiferous lobule; SPC, Spermatocyte; SPG, Spermatogonia, and SPT, spermatid

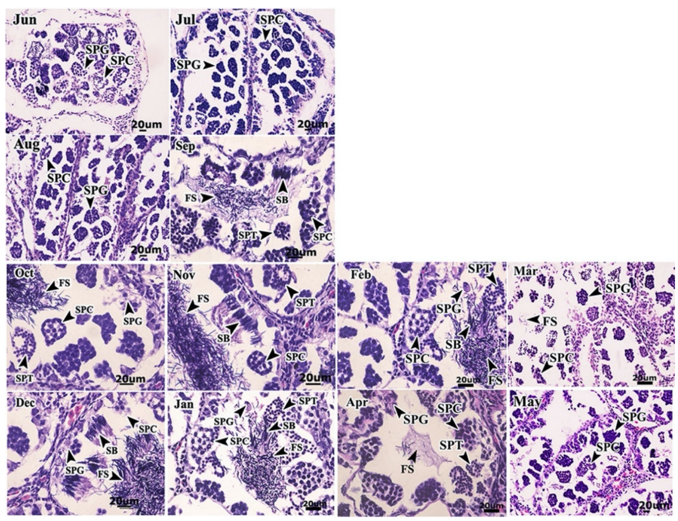

Fig. 5 Spermatogenic events during an annual reproductive cycle, from June to December (2012) and from January to May (2013) of *L. isanensis*
Note: FS, Free sperm; SB, Sperm bundle; SPC, Spermatocyte, SPG, spermatogonia and SPT, spermatid. Jan– Dec denoted January to December, respectively

Annual changes in plasma testosterone

The testosterone profile of male *L. isanensis* is presented as mean ± SD in Fig. 6. The plasma testosterone levels of male *L. isanensis* were low from March to August, gradually increased in September and October, and thereafter rapidly surged from November until February of the next year.

The mean rank of the plasma testosterone levels was significantly different among month groups (Kruskal-Wallis H test, $\chi^2 = 118.909$, $p = 0.0001$). The mean rank between each two month groups (Mann Whitney U test) in each pair of months was compared. The plasma testosterone levels in January (115.467±1.726 ng/mL) and February (116.889±1.054 ng/mL) were little significantly different from each other (U = 34.000, $p = 0.048$), but the levels in both January and February showed significant differences from those in March (35.091±0.831 ng/mL), April (27.778±4.868 ng/mL), May (25.038±0.900 ng/mL), June (24.400 ng/mL), July (22.100±4.185 ng/mL), August (23.167±0.753 ng/mL), September (34.071±1.817 ng/mL), October (36.091±0.700 ng/mL), November (95.600±1.075 ng/mL) and December (102.273± 2.150 ng/mL) (all U = 0.001, all $p = 0.001$). The testosterone level in March was not significantly different from the levels in September (U = 53.500, $p = 0.202$), but it was significantly different from the levels in April (U = 18.000, $p = 0.016$), May (U = 0.001, $p = 0.001$), June (U = 0.001, $p = 0.001$), July (U = 0.001, $p = 0.001$), August (U = 0.001, $p = 0.001$), or October (U = 24.000, $p = 0.016$), November (U = 0.001, $p = 0.001$), and December (U = 0.001, $p = 0.001$). The plasma testosterone level in April showed no significant difference from that in May (U = 38.000, $p = 0.277$) and June (U = 21.500, $p = 0.053$), whereas it differed significantly from the levels observed in July (U = 2.000, $p = 0.001$), August (U = 3.500, $p = 0.003$), September (U = 25.000, $p = 0.016$), October (U = 10.000, $p = 0.002$), November (U = 0.001, $p = 0.001$), and December (U = 0.001, $p = 0.001$). In May, the testosterone level was not significantly different from that in June (U = 38.000, $p = 0.159$), but it showed significant differences from the levels observed in July (U = 0.001, $p = 0.001$), August (U = 4.000, $p = 0.001$), September (U = 0.001, $p = 0.001$), October (U = 0.001, $p = 0.001$), November (U = 0.001, $p = 0.001$), and December (U = 0.001, $p = 0.001$). In June, the testosterone level showed no significant difference compared to the levels in July (U = 33.000, $p = 0.218$) and August (U = 27.000, $p = 0.792$), but it was significantly different from the levels in September (U = 8.5000, $p = 0.001$), October (U = 1.000, $p = 0.001$), November (U = 0.001, $p = 0.001$), and December (U = 0.001, $p = 0.001$). The plasma testosterone level in July was significantly different from the levels in August (U = 11.500, $p = 0.042$), September (U = 0.001, $p = 0.001$), October, November, and December (all U = 0.001, all $p = 0.001$). The plasma testosterone level in August was significantly different from the levels in September, October, November, and December (all U = 0.001, all $p = 0.001$). In September, the testosterone level showed a significant difference from the levels in October (U = 20.000, $p = 0.001$), November (U = 0.001, $p = 0.001$), and December (U = 0.001, $p = 0.001$). In October, the testosterone level differed significantly from the levels in November and December (all U = 0.001, all $p = 0.001$). In November, the plasma testosterone level showed significant difference from that in December (U = 0.001, $p = 0.001$).

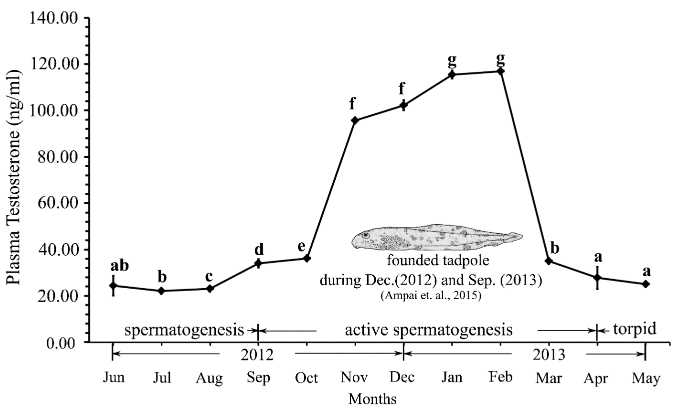

Fig. 6 Annual changes of plasma testosterone in *L. isanensis*. The same small alphabet above the standard deviation of each month indicated a nonsignificant difference between each two months at $p < 0.05$. All months, $n = 10$; Jan–Dec denoted January to December, respectively

Discussion

We might speculate that both environmental low temperature and high relative humidity (ranges of 19°C–21°C and 80–84 percent, respectively) during October to February, rather than rainfall, could be factors triggering the active reproduction of *L. isanensis* habited in tropical zone. However, Rastogi et al. (2011) stated that depending on the species, the most important environmental cues in controlling the anuran reproduction may be environmental temperature and another one is seasonal rainfall, which creates high humidity and a favorable spawning habitat.

Amphibians are generally seasonal breeders (Huang et al., 1996). In species with continuous spermatogenic cycles, the stages of spermatogenesis occur throughout the year regardless of whether or not breeding is seasonal (Rastogi et al., 2011); free sperm are generally produced throughout the year, and the testis always contains spermatic cell nests (Saidapur, 1983). In contrast, some anuran species display a discontinuous type of spermatogenetic cycle in which spermatogenesis is either impaired or completely interrupted during part of the year when the harsh weather sets in or sometimes even in summer (Rastogi et al., 2011). Loft (1974) and Saidapur (1983) considered that the spermatogenic cycle of amphibian species that have free sperm in the seminiferous lobules is of the continuous type. In this study, it was exhibited that from September until February, *L. isanensis*, the Isan big-headed frog, had abundant sperm bundles and free sperm in the lumen of its seminiferous lobules; fewer free sperm were present during March to April, but during May to August, spermatogonia and spermatocytes were present within the germinal cysts in the absence of sperm bundles and free sperm. Thus, the reproductive cycle of *L. isanensis* inhabiting areas next to hillside streams on Phu Luang Mountain at an elevation of 500 meters above sea level (17°03'40.0"N, 101°31'09.3"E), Thailand does not belong either to the continuous or to the discontinuous type of spermatogenic cycle. Huang et al. (1996) demonstrated that *Bufo bankorensis* inhabiting areas along the Honken River in DehKen (24°10'N, 120°43'E), Taichung City, in central Taiwan had free sperm in its seminiferous tubules throughout the year except in April. Additionally, the intensity of spermatogenesis was significantly higher during the breeding period than in the non-breeding period, indicating that while *B. bankorensis* belongs to the potentially continuous breeder group, it does have a distinct breeding period.

Testosterone is well-known as a regulator of spermatogenic activity (Rastogi et al., 2011). The present study showed that tropical male *L. isanensis* exhibit pronounced changes in plasma testosterone levels; testosterone levels increased gradually from September and abruptly in November until February of the following year and thereafter decreased gradually from March to August. Testosterone levels were not found to fluctuate significantly throughout the year in the tropical ranid, *A. larutensis* (Emerson and Hess, 1996). Testosterone levels also were not found to fluctuate during the annual cycle in the Mexican leaf frog, *P. dacnicolor* (Rastogi et al., 1986). The decrease in testosterone level was closely associated with the end of breeding, and the beginning of new spermatogenesis events occurred with the increase in testosterone level (Rastogi et al., 1997). A significant variation in testosterone level was observed that depended on the phase of the reproductive cycle; the pre-reproductive, reproductive, and post-reproductive phases occurred in January to February, in March to June, and in October to December, respectively, in the green frog *R. esculenta* (Raucci et al., 2007). The reproductive strategy of tropical *L. isanensis* was categorized as including a reproductive phase from September until February and a non-reproductive phase from March until August. Seasonal variations in plasma testosterone levels have been monitored in many amphibians, including *R. catesbeiana* (Licht et al., 1983), *R. perezi* (Delgado et al., 1989), *R. rugosa* (Ko et al., 1998), and *B. japonicus* (Itoh et al., 1990). The plasma testosterone level increased and was highest during the breeding season (Pierantoni et al., 1984), but it decreased during the non-breeding season when new spermatogenesis events occurred (Itoh et al., 1990). In *R. esculenta*, the lowest levels of testosterone were observed in summer during the period in which proliferation of spermatogonia and formation of spermatocytes occurred (Rastogi et al., 1976). The Mexican leaf frog, *P. dacnicolor*, breeds continuously, but its testosterone level does not fluctuate (Rastogi et al., 1986). These observations suggest that seasonal variations in plasma testosterone levels are not always associated with active spermatogenesis or with the breeding season (Ko et al., 1998).

Ampai et al. (2015) found egg masses of *L. isanensis*, the Isan big-headed frog, containing 30–40 ova in September and December and also found tadpoles in December at the same site as the egg masses. It was speculated that the breeding season of tropical *L. isanensis* occurs from September (the late rainy season) until December (the early cold season). The results of our study exhibited that the plasma testosterone levels in this species increased gradually in September and October and then abruptly peaked from November until February, corresponding well with the high spermatogenic activity during these months. Inactive testes and lower mass of luminal free sperm occurred concomitantly with low levels of testosterone from March until April. Furthermore, from May until August, no sperm bundles or free sperm were present in the lumen, and only germinal types of spermatogonia and spermatocytes were contained in the germinal cysts, coincident with the decrease in the testosterone level. Thus, in *L. isanensis*, spermatogenic activity is dependent on testosterone-dependent regulation.

The changes in the testicular morphology of male *L. isanensis* observed in this study could be interpreted as indicating the occurrence of an active testicular phase from September to February and an inactive testicular phase occurring from March to May to June to August. Rastogi et al. (2011) stated that the pattern of the male reproductive cycle is based specifically on testicular activity, including both spermatogenesis and steroidogenesis.

In the present study, the reproductive pattern of male *L. isanensis* was conclusively demonstrated to be of a potentially continuous spermatogenic type or an associated reproductive pattern.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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