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STUDIES ON ANNUAL ACTIVITY CYCLE OF GONADS AND BREEDING BEHAVIOR OF THE ENDANGERED FROG, *Rana leptoglossa* (Cope, 1868)

[1]Saha, B. K. & [2]Gupta, B. B. P.
[1]Department of Zoology, Birjhora Mahavidyalaya, Bongaigaon- 783380, Assam, India
[2]Environmental Endocrinology Laboratory, Department of Zoology, North-Eastern Hill University, Shillong – 793022, India

ABSTRACT
Keeping in view the scarcity of information as well as the endemic and endangered status of *R. leptoglossa*, studies were conducted on breeding biology of the frog at Kakoijana Reserve Forest (KRF), Bongaigaon, Assam, India. The gonado-somatic index (GSI) and gonadal histology of both the sexes of the frog were studied during each month at an interval of 30 days during three consecutive years. Observations were also made on its breeding behavior (e.g., mating call, pair formation, mounting, amplexus and spawning.). The male GSI increased and decreased with the increasing (January to June) and decreasing daylength (July to December), respectively. The testicular histology exhibited a circannual activity cycle with the quiescent phase in January and February, the progressive phase during March and April, breeding phase during the months of May to August, and the regressive phase during the months of September to December. The female GSI was found to be the minimum during the month of January, increased gradually and significantly through the months of February and March, reached a peak in the month of April, and thereafter the GSI declined significantly through the months of May/June, July/August, September/ October, and November/December. The ovarian histology of *R. leptoglossa* exhibited a circannual activity cycle with the quiescent phase during the months of September to December, the pre-spawning phase in the months of January and February, the spawning phase during the months of March to May and the post-spawning phase during the months of June to August. The breeding behavior of *R. leptoglossa* seems to suggest that the initiatives for reproduction is taken by the male frog that makes mating calls and aggressive display followed by slow and submissive courtship movement by the female leading to the mounting by the male, amplexus, spawning by the female and milt release by the male.

KEYWORDS: Ranidae, gonado- somatic index (GSI), testis, ovary, amplexus, spawning and milt, breeding cycle, reproduction.

INTRODUCTION

An essential attribute of any surviving species or population is the ability to produce offspring (Duellman and Trueb, 1994; Sarah *et al*., 2009). In amphibians, successful reproduction depends on the location of potential mates (Wells, 2007), stimulation of mates (Wells, 2007; Mellov *et al*., 2010), and selection of breeding site (Kaefer *et al*., 2007). In nature, amphibian breeding activity is accelerated by several environmental factors such as increasing temperature (Saidapur and Hoque, 1995), rainfall (Lynch and Wilczynski, 2005), photoperiod (Saidapur, 1989), pool desiccation (Lind *et al*., 2008), food supply (Girish and Saidapur, 2000), and pond hydrology (Ryan and Winne, 2001; Hagman and Shine, 2006).

In Western Ghats of India, the breeding behavior has been well studied in *Polypedates maculates* (Mohanty and Dutta, 1986), *Rana tigrina* (Dutta and Mohanty, 1990), *Polypedates maculates* (Dutta *et al*., 2001) and *Rhacophorus lateralis* (Dinesh *et al*., 2010). *Ramanella montana* has been reported to lay its eggs in holes of tree trunks (Krishna *et al*., 2004). In case of the shrub frog, *Philautus femoralis* (Bahir *et al*., 2005) and the ground dwelling frog, *Raorchestes resplendens*, froglets develop directly without any tadpole stages (Biju *et al*., 2010). In North Eastern India, breeding behavior has been studied in *Rana limnocharis* (Roy, 1990), *Hyla annectans* (Ao and Bordoloi, 2001), *Chirixalus simus* (Deuti, 2001a, b), *Polypedates leucomystax* and *Rhacophorous bipunctatus* (Iangrai, 2007), *Hylarana humeralis* (Bortamuli *et al*., 2010). There are few reports on the histology of testis and ovary of *Rana cyanophlyctis*, *Rana hexadactyla*, *Bufo melanostictus* (Saidapur, 1989), *Rana curtipes* (Gramapurohit, 2004) and *Rana cyanophlyctis* (Saidapur and Nadkarni, 1975; Pancharatna and Saidapur, 2009). Though the North Eastern India possesses a very rich diversity of amphibians including several endemic and endangered species, there is paucity of information on the breeding behavior and seasonal changes in the histology of gonads. The breeding behavior of the tree frogs, *Polypedates leucomystax* and *Rhacophorous bipunctatus* has been established in Meghalaya (Iangrai, 2007).

A variety of orientation cues has been suggested for the migration to the breeding site by the adult amphibians (Ishii *et al*., 1995). Location of mates in amphibians is achieved by visual cues (Summers *et al*., 1999), olfactory cues (Kikuyama, 2005), auditory cues (Simmons, 2004), or tactile means (physical contact of the male by the female) or a combination of these (Wells, 2007). Selection of the mate depends upon body size, number of nights of calling per male, location and nature of calling sites (Welch *et al*., 1998; Mitchell, 2001; Wells, 2007; Sarah *et* *al.*, 2009). It has been reported that the sites where eggs are deposited can be influenced by the physical features of the breeding sites as well as by the presence of predators, competitors and communal nesters (Magnusson and Hero, 1991). Various studies have suggested that female anurans select oviposition sites based on factors such as water depth, water temperature, water pH, or absence of predators (Hadded and Prado, 2005).

Rana leptoglossa (Synonym *Hylarana leptoglossa*) is one of the endemic and endangered anuran amphibian species found in Assam, Mizoram and Meghalaya (Zoo Outreach Organization, 2001; Saha and Gupta, 2007). It has also been listed as near-threatened globally (IUCN/SSC-CI/CABS, 2004). In order to take appropriate measures for conservation of any rare/endangered species, a sound knowledge of its breeding biology is essential. But so far no attempt has been made to study the annual activity cycle of gonads and breeding behavior of this species. Therefore, it was thought worthwhile to investigate the annual activity cycle of gonads and breeding behavior of this endangered species.

MATERIALS AND METHODS

In order to know the circannual variations in gonadal activity, gonado-somatic index (GSI) of males and females were calculated. For the calculation of GSI, four adult male and four adult female frogs were collected during middle of every month from Kakoijana Reserve Forest (KRF), Bongaigaon, Assam, and brought to the laboratory for further processing. The frogs were weighed and killed by over-anesthesia using chloroform, testis and ovary were rapidly removed, and the weight of the gonads (testis/ovary) of each frog was recorded with the help of an electronic balance (Sartorius CP 225D/CE). The GSI of males and females were calculated by taking total weights of the gonads (TWG) divided by total weight of the frog (TWF) multiplied by 100 [TWG X 100/TWF].

All studies under captive conditions were conducted on the frog maintained under an enclosure measuring 1m X 1m X 1m in dimension with a water pool, and hiding places were constructed for maintaining 4-6 pairs of *Rana leptoglossa* under natural climatic conditions. The frogs were fed with insects, earthworms, and insect larvae *ad libitum* during the captivity.

In order to study the breeding behavior, observations were made on courtship, mounting/amplexus and spawning of the frog, *R. leptoglossa* in three consecutive years (2005, 2006, and 2007) during the months of March to August) at the breeding sites in the KRF. Further, courtship, mounting/amplexus and spawning were documented with the help of photographic camera under both natural habitat and captivity. The duration when the breeding pairs remained in amplexus was noted down and the environmental parameters such as temperature and relative humidity were also recorded with thermometer and hygrometer, respectively. Water temperature and water pH were recorded using thermometer and pH indicator paper (Universal indicator pH 1-10, MERCK), respectively.

To study the histology of the testis and ovary, adult male and adult female frogs were collected from the K.RF every month starting from January to December, and brought to the laboratory. Since the frog was not available in its natural habitat during the months of September to February (hibernation phase), adult male and female frogs were collected in good numbers from the reserve forest and maintained under captive conditions for the histological studies of testis and ovary during the months of September to February. The frogs were killed every month at an interval of 30 days under over-anesthesia, testis of the males and ovaries of the females were dissected out and immediately fixed in Bouin's fluid for 24 hrs. The gonads were then processed following established protocol for the preparation of histological slides. Photographs of the histological sections were taken with the help of an Image Analysis System (Leica Image Analyzer DM 1000). The images of the testis and ovary were captured at 10 X and 2.5 X magnifications, respectively.

The data were analyzed statistically with the help of Regression Analysis and one way analysis of variance (ANOVA) using a software (Systat 11.0).

RESULTS

Annual cycle of GSI in males and histology of testis

The data on GSI in male *R. leptoglossa* is presented in Tables 1 & 2. In case of male *Rana leptoglossa*, the GSI was found to be minimum in the month of January. The GSI increased gradually but significantly through the months of February to May and reached a peak in the month of June. Thereafter, the GSI declined significantly through the months of July/August, September/October, and November/December. Regression analysis of the data indicated a significant positive correlation between male GSI and temperature ($r = 0.75$), daylength ($r = 0.94$) and rainfall ($r = 0.80$) (Table 2 and Fig. 1).

The monthly variations in the histology of testis of *Rana leptoglossa* are presented in Plate 1. The seminiferous tubules contained large numbers of only spermatogonial cells during the months of January and February. However, during the months of March and April, seminiferous tubules were filled with spermatogonia, spermatocytes, spermatids and spermatozoa. Slender and filamentous spermatozoa were found in bundles attached to Sertoli cells. During the months of May to August, seminiferous tubules were filled with bundles of mature spermatozoa and lesser numbers of spermatogonia, spermatocytes and spermatids. During the months of September and October, the numbers of spermatozoa declined gradually due to degeneration of sperms, and the spermatids were observed in bundles. During the months of November and December, the testis contained only small number of spermatozoa along with few spermatogonia but no spermatids.

Annual cycle of GSI in females and histology of ovary

The data on GSI in female *R. leptoglossa* is presented in Tables 1 & 3. In case of female *R. leptoglossa*, GSI was found to be the minimum during the month of January. The GSI increased gradually and significantly through the months of February and March, and reached a peak in the month of April. Thereafter the GSI declined significantly through the months of May/June, July/August, September/ October, and November/December. Regression analysis of the data indicated positive and significant correlation between female GSI and temperature ($r = 0.52$), daylength ($r = 0.70$) and rainfall ($r = 0.42$) (Table 3 and Fig. 2).

Monthly variations in the histology of the ovary of *Rana leptoglossa* are presented in Plate 2. During the months of January and February, the ovarian histology revealed the presence of numerous oogonial mother cells (OG), primary oocytes (PO) and secondary oocytes (SO) in the ovarian follicles (Plate 2). During the months of March to May, the ovary was found to contain several OG, PO, SO and mature ova (OV). During the months of June to August, the ovary contained PO, SO and ova of smaller sizes (Plate 2). During the months of September and October, the histology of the ovary revealed the presence of only oogonial mother cells, and the ovary was depleted of ova. During the months of November and December, the ovary was filled with only oogonial mother cells, which give rise to PO.

TABLE 1. Circannual variations in the gonado-somatic index (GSI) of male and female *Rana leptoglossa*

MONTHS	GSI of male *R. leptoglossa* (%)	ANOVA (Level of significance)	GSI of female *R. leptoglossa* (%)	ANOVA (Level of significance)
January	0.172 ± 0.003*	0.917	1.19 ± 0.04*	0.035 [a]
February	0.261 ± 0.006	0.001 [c]	4.59 ± 0.10	0.001 [c]
March	0.317 ± 0.003	0.001 [c]	6.37 ± 0.00	0.001 [c]
April	0.361 ± 0.003	0.001 [c]	6.39 ± 0.01	1.000
May	0.385 ± 0.008	0.099	5.89 ± 0.01	0.005 [b]
June	0.467 ± 0.003	0.001 [c]	5.61 ± 0.06	0.563
July	0.409 ± 0.007	0.001 [c]	4.27 ± 0.05	0.001 [c]
August	0.400 ± 0.002	0.988	3.87 ± 0.24	0.056
September	0.229 ± 0.00	0.001[c]	2.82 ± 0.05	0.001 [c]
October	0.227 ± 0.00	1.000	2.74 ± 0.08	1.000
November	0.190 ± 0.011	0.001 [c]	1.65 ± 0.02	0.001 [c]
December	0.184 ± 0.004	0.999	1.61± 0.01	1.000

* All values are expressed as mean ± standard error (S. E.); N=4.
[a, b, c] Differ significantly from the value of preceding month: $p < 0.05$, 0.01 and 0.001, respectively.

TABLE 2. Correlation between the male gonado-somatic index (GSI) and climatic factors

Temperature vs. male GSI	Daylength vs. male GSI	Rainfall vs. male GSI	Relative humidity vs. male GSI
r = 0.75 ($p < 0.05$)	r = 0.94 ($p < 0.05$)	r = 0.80 ($p < 0.05$)	r = - 0.14 ($p < 0.05$)

r = Correlation coefficient.

TABLE 3. Correlation between the female gonado-somatic index (GSI) and climatic factors

Temperature vs. female GSI	Daylength vs. female GSI	Rainfall vs. female GSI	Relative humidity vs. female GSI
r = 0.52 ($p < 0.05$)	r = 0.70 ($p < 0.05$)	r = 0.42 ($p < 0.05$)	r = - 0.62 ($p < 0.05$)

r = Correlation coefficient.

Breeding behavior under natural and captive conditions

Observations were made on courtship, mating and spawning of the frog, *Rana leptoglossa*, and the photographs were presented in Plates 3 & 4. The reproductive behaviors were exhibited with the arrival of the first rain in March and continued till the month of August. The frogs were found to emerge from hibernation during the month of March and started to migrate towards the breeding sites. The males were observed to be first to arrive at the breeding sites, were found hiding in between the tall grasses and emitted advertisement calls/mating calls, which attracted the females to come out from their hiding places to the breeding grounds/sites. The males were found sitting on the edges of the water bodies or on the submerged aquatic vegetation after sunset at the breeding sites and making advertisement calls. The breeding males exhibited a high posture with inflated lungs and stretched limbs over water, with the toes and belly under water, the head held up at a slight angle exposing the bright white throat. Maintenance of such a posture by the males was observed even when they were not actively calling. Other territorial males (satellite males) entering the breeding ground were observed to maintain a low posture with only the top of the head and eyes above the surface of the water. Aggressive behavior was also exhibited by the sexually active males. When a male of the same breeding territory was found to enter into the area of another individual, it was attacked by the resident male. When the female approached the breeding site, the male moved towards the female and amplexus took place after the sunset. For selecting the male partners, females slowly moved in to the male territories with a low posture similar to that of the satellite males. After reaching close to the male, the female was found to become stationary. Then a male climbed on the back of a suitable female and clasped her in axillary amplexus. The process of courtship and mounting was found to be common under both natural and captive conditions (Plates 3 & 4). The pair remained in amplexus for 1-2 hrs or sometimes even longer depending upon the ambient conditions. After mounting/mating the female frog released the eggs in water which became attached to twigs or aquatic vegetation by thick coat of jelly layer (Plates 3 & 4). The male *R. leptoglossa* simultaneously secreted its milt (fluid containing sperms) over the eggs to fertilize them. The courtships and mountings were observed invariably during the second half of April and first half of May under both natural and captive conditions. Once the spawning was over, both male and female frogs moved away from each other as well as from the spawning site showing no signs of parental care. It was observed that there was always 10 to 70 cm deep water below the spawns.

DISCUSSION

The present findings suggest that the male *Rana leptoglossa* exhibits seasonal variations in its GSI. The GSI of the male frog was minimum during the month of January with minimum daylength, increased gradually but significantly through the months of February to May with increasing daylength, reached a peak in June with maximum daylength, and thereafter declined gradually and significantly through the months of July to December with the decreasing daylength. Thus, the male GSI seems to increase and decrease with the increasing (January to June) and decreasing daylength (July to December), respectively. Further, though the regression analysis indicated significant positive correlation between the male GSI and temperature (r = 0.75), daylength (r = 0.94) and rainfall (r = 0.80), the correlation coefficient suggested a stronger correlation of the male GSI with the daylength than that with temperature or rainfall. No significant correlation was found between the male GSI and relative humidity (Tables 1 & 2). Though the ambient temperature also increases and decreases with the increase and decrease in daylength (photoperiod), our observation did not indicate any parallel increase or decrease in male GSI in relation to the ambient temperature or rainfall/relative humidity which failed to exhibit any circannual pattern. On the basis of present findings, we suggest that the circannual rhythm of male GSI of *R. leptoglossa* is regulated directly by the seasonal variations in the daylength which is highly predictable as compared to temperature and rainfall which were highly variable and unpredictable on the annual time scale. It seems that the male frog depends solely on seasonal variations in daylength to programmes its circannual cycle of testicular activity.

The histology of testis of *R. leptoglossa* indicated the presence of large numbers of only spermatogonial cells during the months of January and February, presence of moderate number of spermatozoa along with other cells (i.e., spermatogonia, spermatocytes and spermatids) during the months of March and April, large bundles of mature spermatozoa and lesser numbers of other cells during the months of May to August, degenerating spermatozoa and bundles of spermatids in the months of September and October, and only small number of degenerating spermatozoa along with few spermatogonia but no spermatids in November and December (Plate 1). These observations clearly indicate that the testicular activity exhibits a circannual cycle with the quiescent phase in January and February, the progressive phase during March/April, the breeding phase during the months of May to August, and the regressive phase in the months of September to December. The presence of normal spermatozoa in the testis during the months of March to August seems to indicate that the male frog is sexually active starting with its emergence from hibernation in March till its migration towards hibernation after August. It is important to mention that the duration of active spermatogenesis coincides with the non-hibernating period of the frog.

The GSI of female *R. leptoglossa* was minimum during the month of January, increased gradually and significantly through the months of February and March, reached a peak in the month of April and thereafter the GSI declined significantly through the months of May to December. Regression analysis indicated positive and significant correlation between female GSI and temperature (r = 0.52), daylength (r = 0.70) and rainfall (r = 0.42) (Table 3 & Fig. 2). The correlation coefficient indicates that the correlation between the GSI and environmental factors (i.e., temperature, daylength and rainfall) was stronger in males as compared to that in females (Tables 2 & 3). Further, the observations on ovarian histology for the two consecutive years revealed the presence of numerous oogonial mother cells (OG), primary oocytes (PO) and secondary oocytes (SO) in the ovarian follicles in January and February; several OG, PO, SO and mature ova (OV) during the months of March and May; PO, SO and ova of smaller sizes in the months of June to August; and only oogonial mother cells in the months of September to December (Plate 2). These findings seem to suggest that the female *R. leptoglossa* exhibits a circannual cycle of ovarian activity with the quiescent phase during the months of September to December, the pre-spawning phase in the months of January and February, the spawning phase during the months of March to May and the post-spawning phase during the months of June to August. Unlike in males where normal spermatozoa were present in the testis from March to August, the spawning phase of the females was restricted only to March to May. These findings seem to suggest that while the males are ready for breeding throughout the non-hibernation phase, the females restrict the breeding phase of this species by restricting the spawning phase only during the months of March to May.

Observations on the breeding behavior of *R. leptoglossa* seem to suggest that the initiatives for reproduction is taken by the male *R. leptoglossa* that makes mating calls and aggressive display which are followed by slow and submissive courtship movement by the female. This movement of the female leads to the mounting by the male resulting in amplexus leading to spawning by the female and milt release by the male.

It is important to mention that we did not find any difference between the annual activity cycle (i.e., hibernation phase, emergence from hibernation, breeding phase and migration to hibernation sites), the seasonality in breeding cycle as well as the breeding behavior of *R. leptoglossa* studied under its natural habitat and captivity. Therefore, our findings seem to suggest that the endangered and endemic frog, *R. leptoglossa* can be easily maintained and allowed to breed under captive conditions for its *ex situ* conservation.

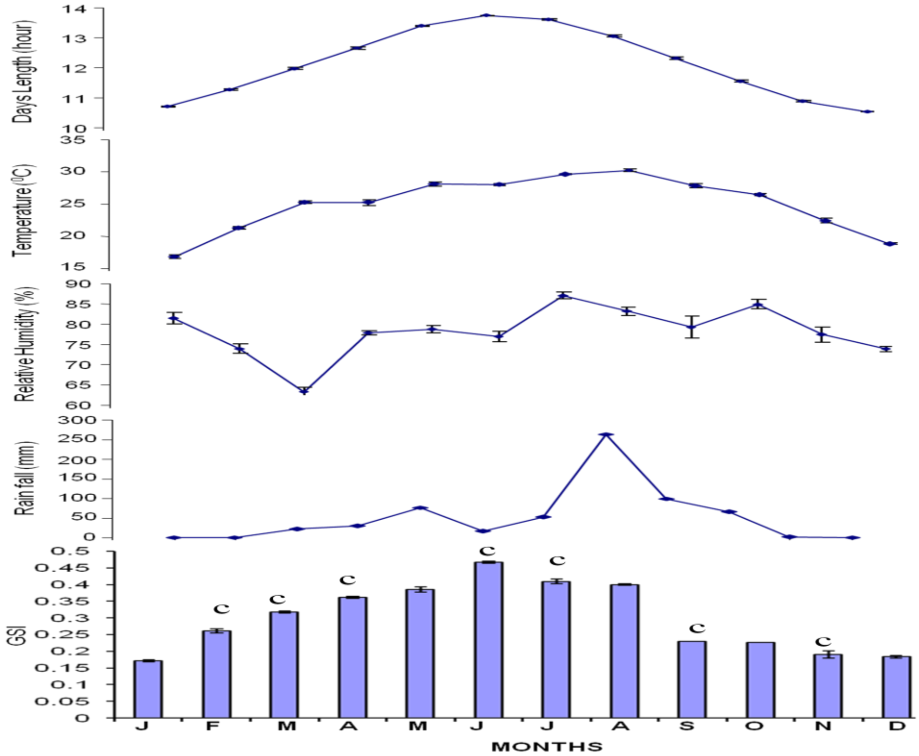


FIGURE 1. Monthly variations in GSI of male *Rana leptoglossa* in relation to climatic factors.
[a, b, c] Differ significantly from the value of preceding month: $p < 0.05$, 0.01 and 0.001, respectively.

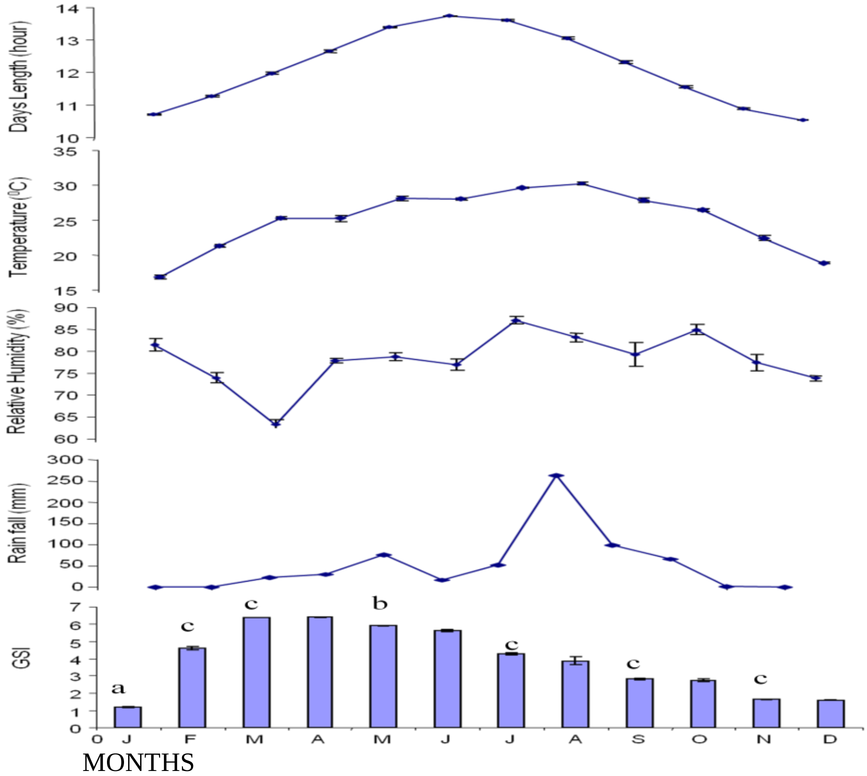


FIGURE 2. Monthly variations in GSI of female *Rana leptoglossa* in relation to climatic factors.
[a, b, c] Differ significantly from the value of preceding month: $p < 0.05$, 0.01 and 0.001, respectively.

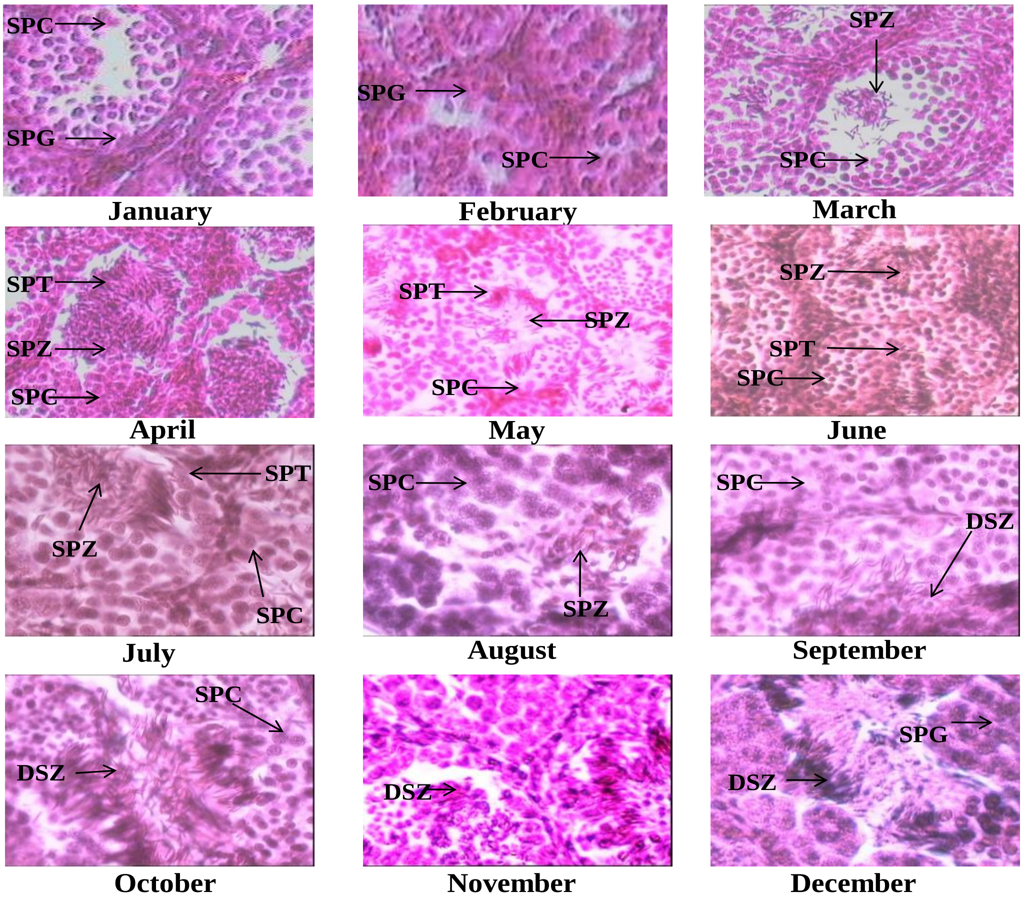


PLATE 1. Circannual variations in the testicular histology of *Rana leptoglossa*.
SPG= Spermatogonia; SPC= Spermatocytes; SPT= Spermatids; SPZ= Spermatozoa, and DSZ= Degenerating spermatozoa; Magnification = X10.

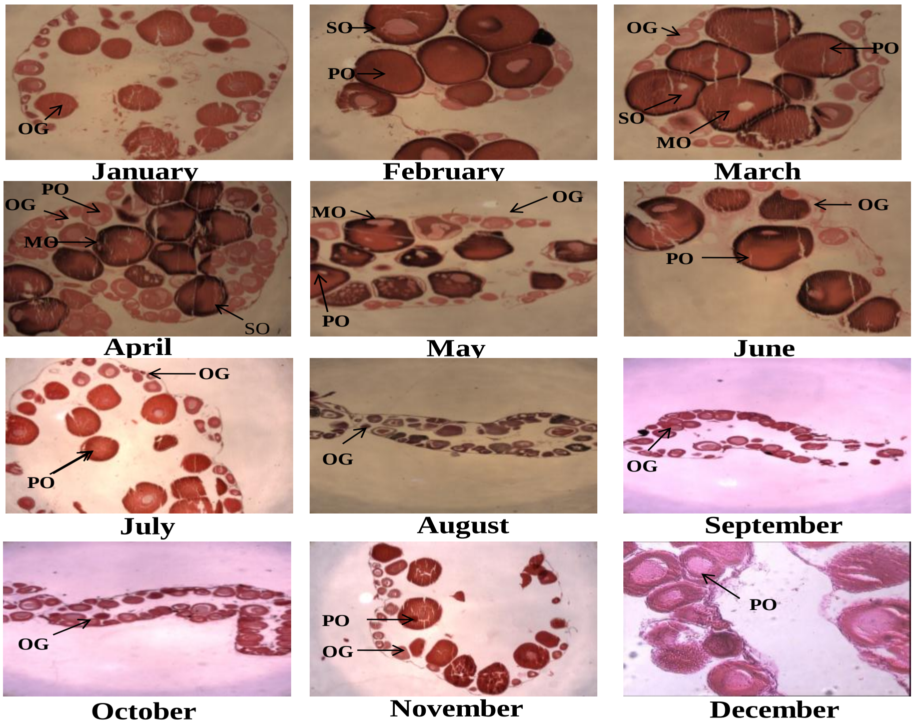
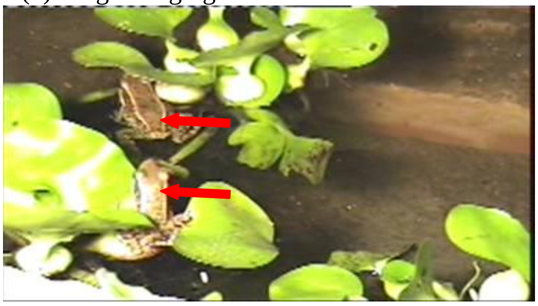


PLATE 2. Circannual variations in the ovarian histology of *Rana leptoglossa*.
OG= Oogonium; PO= Primary oocytes; SO= Secondary oocytes and MO= Mature ova; Magnification= X 2.5.

(a) Hibernating frog

(b) Frog emerging after hibernation

(c) A male frog with vocal sac



(d) A male following the female

(e) A pair in amplexus

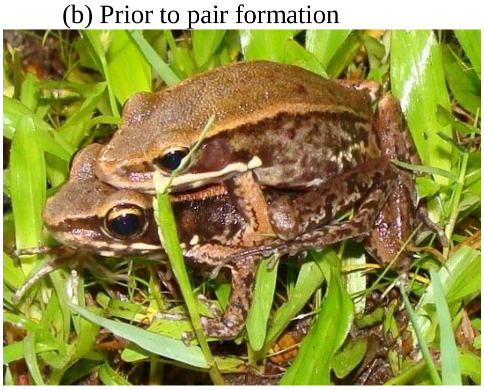
(f) Spawning frog in amplexus

PLATE 3: Breeding behaviors of *Rana leptoglossa* under natural habitats.

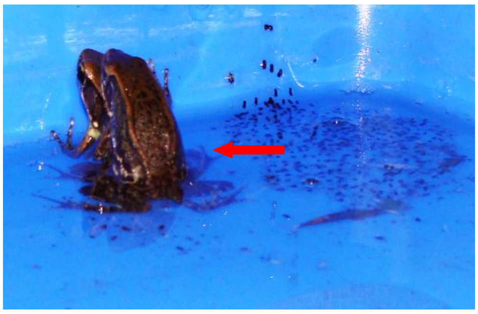
(a) Frog emerging after hibernation

(b) Prior to pair formation

(c) Amplexed frogs



(d) Amplexed frogs



(e) Spawning by amplexed frog

(f) Spawn of *Rana leptoglossa*

PLATE 4. Breeding behaviors of *Rana leptoglossa* under captive conditions.

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