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Seasonal and daily changes in thyroid activity of male common toad, *Bufo melanostictus* in Imphal valley, India

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Abstract
Thyroid hormones are involved in various thyroid activities in higher and lower vertebrates however, the activities are not clearly known. Studies on seasonal changes in glandular and circulating levels of thyroid hormones in different species have revealed varying patterns of changes. Apparent circadian rhythms in plasma and thyroid contents of T_4 and T_3 have been reported. However, all these findings are limited to temperate zone species and no corresponding information is available on any sub-tropical anuran. Therefore, the purpose of the present study was to estimate seasonal changes in thyroid activity in relation to reproductive events in a sub-tropical male toad, *Bufo melanostictus***.** Changes in circadian level of T_3 and T_4 were monitored to correlate data with daily and seasonal metabolic events in this species. Thyroid histology was studied microscopically; radioimmunoassay of T_3 and T_4 was studied. In the present study, it was observed that thyroid activity in male toad was maximum during February and minimum during August. The results of the present study on *Bufo melanostictus* also showed that circulating levels of thyroid hormones, T_3 and T_4, both are minimum during June/July. Progressive increase in plasma T_3 and T_4 levels of *Bufo melanostictus* was associated with progressive increase in gonado somatic index of male toad between August and December. Plasma T_3 and T_4 both had slight depression during December-January which was followed by continued increase in T_4 in males until March/April. High level of circulating T_3 and T_4 in association with maximum activity of thyroid follicles probably indicates to the fact that high synthesis and release of thyroid hormones during this period is related to emergence of toads from hibernation.

Keywords: male toad, thyroid hormones, circadian level, gonado somatic index

Introduction
In higher vertebrates thyroid hormones have been shown to be crucially involved in a number of vitally important physiological processes such as daily food uptake, oxidative metabolism, haemopoiesis, migration, moult and reproduction, etc. (Hulbert, 1985; Kar and Chandola, 1985; Nicholls *et al*. 1988; Thapliyal and Gupta, 1989) [4, 13, 21]. In lower vertebrates although several studies based on histometric analysis and/or radioimmunoassay quantification of thyroid hormones in the follicles/glands or in circulation have revealed profound alterations in thyroid activity (Rosenkilde, 1982; Kar and Chandola, 1985) [16, 4] the precise physiological significance of thyroid hormones in seasonal physiological events of adult individuals is largely obscure. Studies on seasonal changes in glandular and circulating levels of thyroid hormones in different species have revealed varying patterns of changes. Several researchers observed that plasma thyroid hormone levels are elevated during breeding season in fish (Chakraborti and Bhattacharya, 1984) [3], female cobra (Bona-Gallo *et al*. 1980), but some other reported that the levels were higher in a period either before the breeding season as in toads (Rosenkikde and Jorgensen, 1977) [17], frogs (Kuhn *et al*., 1985) [9] or after the breeding season as in male cobras (Bona-Gallo *et al*., 1980). In *Bufo bufo*, plasma T_4 had highest levels in spring and summer (Rosenkilde, 1982) [16]. In contrast, in lizard, *Cnemidophorous sexlineatus* (Seller *et al*., 1982) [18], the highest peak of the plasma T_4 was observed in winter. In *Bufo bufo*, observed a rise in the plasma T_4 around the end of the hibernation period (Rosenkilde, 1982) [16].
An apparent circadian rhythms in plasma and thyroid contents of T_4 and T_3 have also been reported in rat (Jordon *et al*. 1980), immature domestic fowl (Newcomer, 1974; Klandrorf *et al*. 1978; Kuphn *et al*. 1982) [12], duck (Harvey *et al*. 1980), teleostean fishes like brook trout (White and Henderson, 1977) [22], gold fish (Spieler and Noeske, 1981) [19], rainbow trout (Osborn *et al*. 1978) [15], and amphibian like *Rana ridibunda* (Kuhn *et al*. 1983) [11] and temperature acclimated *Bufo viridis* (Kuhn *et al*. 1985) [9]. No circadian variations in plasma T_3 and T_4 were observed in neotenic salamander (Norris *et al*. 1981). In amphibians while definite role of thyroid hormones has been established in larval metamorphosis, their role in seasonal events of adults is poorly understood. Barring *Bufa japonicas* (Tasaki *et al*. 1986) [20] in which circulating levels of thyroid hormones have been measured during a year while other observations were made intermittently and involved fewer values.

Further, information on circadian variations in plasma thyroid hormones is restricted either to specific sexual stage or to temperature acclimated individuals (Kuhn *et al*. 1985) [9]. Further all the information are limited to temperate zone species and no corresponding information is available on any sub-tropical anuran. The purpose of the present study was to estimate seasonal changes in thyroid activity in relation to reproductive events in a sub-tropical male toad, *Bufo malanostictus***.** Changes in circadian level of T_3 and T_4 were monitored to correlate data with daily and seasonal metabolic events in this species.

Materials and Methods

Beginning from May, 1989 to April 1990, during the last week of every month, 5 male (20-40g) toads were collected from the University campus and were brought to the laboratory in aluminium cages. Toads were bled by exposing the heart. Blood was collected by cardiac puncture using heparinized syringe. Blood samples (3 - 4 ml) were spin at 3000 rpm for 20 min and separated and frozen at -20ºC until assayed for T_3 and T_4. During December (prespawning phase), 1989, April (spawning phase) and August (preparatory phase), 1990, 4-5 adults male and female toads were separately bled at 3 hour interval for a total period of 24 hours. Samples were spin, plasma separated and stored at -20ºC until assayed for T_3 and T_4. Following blood collection toads were killed by decapitation. Thyroid regions were fixed in Bouin's fluid for microscopic study.

Thyroid Histology

Tissues were dehydrated and embedded in paraffin wax. Serial sections were cut at 5-6 µm and stained with haematoxylin/eosin. For the assessment of thyroid glands, epithelial cell height (ECH), nuclear diameter of epithelial cell (NDEC) were measured and appearance of follicles, colloid and epithelial cells were evaluated as described by Sonstegard and Leatherland (1976). Follicular appearance and colloidal content of the glands were assessed separately on a 1-5 scale, 1 representing to the uniform follicular form and homogenous non-depleted colloid depleted follicles. The epithelial cell appearance was evaluated on a scale of 1-6. One (1) representing to low profile, cuboidal or squamous epithelium of non-stimulated follicle, 2 representing to cuboidal epithelium, 3 to moderately hyperplastic cuboidal and columnar epithelial cells and stage 5 and 6 to different degrees of columnar forms. Follicular index, colloidal index and epithelial index were calculated on the basis of these assessments. A "thyroid index" was derived for each toad by adding follicular index, colloidal index and epithelial index values. For the calculations of thyroid epithelial cell height (TEH) and nuclear diameter of thyroid epithelial cell (NDTEC), 20 follicles were selected randomly from male toad and in these heights of 40 epithelial cells and their nuclei were measured using ocular micrometer.

Radioimmunoassay of T_3 and T_4

Plasma samples were assayed in duplicates using single antibody procedure in which labelled ^{125}I and unlabelled hormone (standard/unknown samples) couple for binding sites on a specific antibody. Standards, non-specific binding and quantity/quality controls were ran in each assay as per protocol from BARC. Standard solution of thyroid hormones for both T_3 and T_4 RIA were prepared in amphibian ringer (NaCl, 6.6 g; 30 mg; Penicillin G-sodium, Streptomycin sulphate, 50 mg; $NaHCO_3$, 200mg; BSA, 1 g dissolved in 1 litre of double distilled water). In both the RIAs, the used thyroid hormones were of high specific activity (^{125}I-T_3, 300 µci/µg; ^{125}I-T_4, 300-500 µci/µg). Suitable volume (100 µl) of both the hormones were taken. Sample volumes of standard as well as unknown in either assay were fixed at 100 µl. Incubation for both T_4 and T_3 assays were performed at room temperature (24±2ºC) for 75 min and 3 hours respectively. For separation of bound and free fractions, first samples were spin at 3000 rpm for 15 min then 1 ml of 10% chilled polyethylene glycol (dissolved in 1% w/v, NaCl solution) was added; samples were once again spin at 3000 rpm for 15 min and then the supernatant was carefully discarded. Radioactivity present in the bound fraction was determined with 1 min count on Gamma-ray spectrophotometer (ECIL, India) at 56% efficiency. The percent bound fraction (% binding/ 0% binding) was used as responsemeter in both the assays. Standard curves for both T_3 and T_4 were plotted between the log standard hormone concentration on X axis vs logit transformations of percent binding on Y axis. T_3 RIA standard curve covered a range from 0-5ng/ml of T_3 whereas T_4 in plasma were calculated from the standard curve and/or calculated from a PC based RIA program (NIH, U.S.A).

Evaluation of T_3 and T_4 sensitivity

The index of precision (Midgley, Niswender and Rager, 1969) and the detection limit or sensitivity (Ekind, 1974) were determined from the regression curves of both T_3 and T_4 assays.

Reproducibility of Precision

The percent coefficient of variation (% cv) was calculated according to Z, Cekan 1975, to determine the intra and inter assay reproducibility or precision in both the assays. The intra assay reproducibility was made by assaying same sample at different time intervals in four separate assays. Percent coefficient was calculated. Data was analysed using Student's "t" test or by Newman-kuel's multiple range "t" test at 95% confidence limit following analysis of variance (ANNOVA) (Bruning and Kintz, 1977) [1].

Result and Discussion

Seasonal variations in thyroid histology

All the histometric parameters used for evaluating thyroid activity showed a similar pattern of change (Table 1). Based on thyroid index thyroid activity was observed to have progressive increase between August and

February, started declining thereafter, reaching to seasonal minima during August in either sexes. In February, mean values for epithelial cell height, nuclear diameter of epithelial cells and thyroid index were significantly higher ($P<0.05$, $P<0.001$ and $P<0.01$) from their values in the preceding month (Table 1), values for epithelial and colloid index were occasionally significantly different but follicular index did not exhibit any significant alteration (Table 1).

Seasonal changes in circulating levels of thyroid hormones

In the male toads plasma T_3 levels was seasonal maximum during November (1.776 ± 0.163 ng/ml) and minimum during July (0.182 ± 0.05 ng/ml). T_4 levels decreased steadily between November and March. A transient rise occurred between April and May which was followed by rapid decline between May and July. Mean plasma T_3 levels between August and May were significantly higher compared to baseline value (Table 2). Plasma T_4 levels varied between 4.92 ± 0.55 ng/ml in March and 3.04 ± 0.09 ng/ml in June. T_4 levels began rising during July attained higher level in August, increased further between August and November which was followed by drastic and significant ($P<0.05$) decline in December. A resurgent increase in plasma T_4 levels were observed between December and March/April, which was followed by decline between April and June (Table 19). Mean values of T_4 during November, December and between March and May were significantly higher compared to baseline during June (Table 2). Plasma T_3/T_4 ratio was maximum during December (0.472 ± 0.074 ng/ml and minimum in July (0.068 ± 0.022 ng/ml). Between August and February T_3/T_4 ratio was significantly higher compared to baseline value of July (Table 2). ANOVA revealed a significant variations in T_3, T_4 and T_3/T_4 ratio (Table 3).

Circadian variations in circulating levels of T_3 and T_4 in male toads

I- Pre-spawning phase (December, 1989)

Plasma T_3 levels varied during 24 hours with acrophase at 15 hours (0.89 ± 0.099 ng/ml) and bathyphase at 12 hours (0.155 ± 0.016 ng/ml). At 9, 15 and 21 hours mean values for T_3 were also higher and were significantly more compared to base line value at 12 hours (Table 4). Marked circadian variations in plasma T4 levels were significantly low at 12 and 24 hours compared to the values during rest of the periods (Table 4). T_3/T_4 ratio was maximum at (0.175 ± 0.059) at 15 hours and minimum (0.032 ± 0.003) at 18 hours. T_3/T_4 ratios at all hours were significantly higher compared to minimum value obtained at 18 hours (Table 4).

II- Spawning phase (April, 1990)

Apparent fluctuations in mean T_3 levels with its maxima at 18 hours and 3 hours minima at 21 hours. Mean T_3 were significantly higher at all hours when compared with the value obtained at 21 hours (Table 5). Plasma T_4 fluctuated significantly at 6, 18 and 3 hours when compared with the levels picked up at 21 hours (Table 5). T_3/T_4 ratio was maximum at 12 hours (0.172 ± 0.027) and minimum at 6 hours (0.103 ± 0.013 ng/ml). Mean values of T_3/T_4 ratio at 12, 18 and 3 hours were significantly higher compared to the value obtained at 6 hours (Table 5).

III- Preparatory phase (August, 1990)

During August T_3 values did not change markedly although significant alterations with peak value at 6 hours (0.725 ± 0.178 ng/ml) and minimum at 21 hours (0.340 ± 0.020 ng/ml) were observed. A 6 and 24 hours plasma T_3 values were significantly higher compared to the value measured at 21 hours (Table 6). Except that plasma T_4 level was significantly suppressed at 15 hours, the values were fairly high at all points. Mean values plasma T_4 at 12, 18, 24 and 3 hours were significantly different from that obtained at 15 hours (Table 6). No significant alteration across 24 hours was obtained in T_3/T_4 ratio (Table 6). Present results show that thyroid activity in male toads was maximum during February and minimum during August. Progressive increase in thyroid index between August and February was associated with progressive increase in circulating levels of thyroid hormone. This observation although based on qualitative changes in thyroid characteristic like epithelial cell height, nuclear diameter and colloidal status serves as a reliable method to assess thyroidal activity in *Bufo melanostictus*. Histological changes in thyroid follicles as an index of thyroid function has also been used by several workers in different species. Thyroid activity by measurements of epithelial cell height and nuclear diameter of epithelial cells following thiourea treatments has been studied by Hussain and Saidapur 1982. These authors have shown that in the *Bufo melanostictus*, follicular changes following goitrogen treatments were associated with hypertrophy of thyrotroph cells in the adenohypophysis. Present results on *Bufo melanostictus* showed that circulating levels of thyroid hormones, both T_3 and T_4 are minimum during June/July. In the present study, progressive increase in plasma T_3 and T_4 levels of *Bufo melanostictus* was associated with progressive increase in gonado somatic index of the male toads between August and December. Plasma of both T_3 and T_4 had slight depression during December-January which was followed by continued increase in T_4 in males and females until March/April. High level of circulating T_3 and T_4 in association with maximum activity of thyroid follicles probably indicates to the fact that high synthesis and release of thyroid hormones during this period is related to emergence of toads from hibernation. Present observations clearly indicate the existence of circadian oscillation in thyroid activity. It seems that in these mammals as in toads, minimal thyroid activity is indispensible for survival and that the persistence of a circadian cyclicity for T_3 in plasma of non-feeding hibernations may reflect the special metabolic state and food reserve of the animal.

Table 1: Annual changes in thyroid gland characteristics in male toad, *Bufo melanostictus*

Months/ Year 1988	ECH (µm)	NDEC (µm)	Thy. Index	F. index	Epi. Index	C. index
Jan.	14.12 ± 0.21	5.75 ± 0.20	11.75 ± 0.11	3.76 ± 0.07	4.12 ± 0.06	3.89 ± 0.06
Feb.	14.83 ± 0.08^{b}	7.02 ± 0.12_{d}	12.27 ± 0.08^{c}	3.87 ± 0.06	4.28 ± 0.05	4.11 ± 0.11
Mar.	14.04 ± 0.12_{d}	5.74 ± 0.14^{d}	11.62 ± 0.36	3.69 ± 0.22	4.02 ± 0.23	3.76 ± 0.06_{a}
Apr.	13.47 ± 0.35	5.50 ± 0.16	11.09 ± 0.28	3.54 ± 0.31	3.96 ± 0.11	3.59 ± 0.17
May.	11.54 ± 0.25_{c}	4.67 ± 0.22^{b}	10.01 ± 0.19_{b}	3.23 ± 0.12	3.45 ± 0.16^{a}	3.35 ± 0.21
Jun.	12.06 ± 0.20	5.20 ± 0.16	10.13 ± 0.20	3.25 ± 0.10	3.51 ± 0.21	3.36 ± 0.19
Jul.	12.60 ± 0.10_{a}	5.43 ± 0.21	10.58 ± 0.18	3.32 ± 0.13	3.79 ± 0.21	3.47 ± 0.18
Aug.	11.43 ± 0.43^{a}	4.49 ± 0.14_{c}	9.82 ± 0.37	3.18 ± 0.29	3.37 ± 0.03	3.29 ± 0.15
Sep.	12.01 ± 0.18	4.78 ± 0.15	10.37 ± 0.30	3.31 ± 0.20	3.69 ± 0.13^{a}	3.37 ± 0.17
Oct.	12.73 ± 0.12_{b}	5.48 ± 0.24^{a}	10.73 ± 0.37	3.39 ± 0.15	3.86 ± 0.07	3.48 ± 0.29
Nov.	14.76 ± 0.16_{d}	6.81 ± 0.38^{b}	11.91 ± 0.18_{a}	3.76 ± 0.17	4.16 ± 0.06^{b}	3.99 ± 0.06
Dec.	13.85 ± 0.31_{a}	5.61 ± 0.21^{a}	11.30 ± $0._{14c}$	3.67 ± 0.04	3.98 ± 0.04^{a}	3.66 ± 0.16

Values were expressed as Mean+/- SEM; n,40 in each animal in case of ECH, NDEC and others are n, 5.ECH – Epithelial Cell Height; NDEC – Nuclear diameter of epithelial cell; Thy. Index – Thyroid index; F. index – Follicle index; Epi. index – Epithelial index C. index – Colloid index.

a, b, c, d differ from preceding month value at p< 0.05, <0.02, <0.01 and <0.001 respectively (Student's "t" – test).

Table 2: Annual changes in thyroid hormone levels in the plasma of male toad, *Bufo melanostictus*

Months/ Year 1989	T_3 (ng/m1)	T_4 (ng/m1)	T_3/T_4 (ng/m1)	GSI
May.	1.082 ± 0.171^{*}	4.12 ± 0.58^{*}	0.214 ± 0.022	0.322 ± 0.026^{*}
Jun.	0.540 ± 0.040^{c}	3.04 ± 0.09^{c}	0.177 ± 0.013	0.290 ± 0.030^{*}
Jul.	0.182 ± 0.050^{d}	3.12 ± 0.64	0.068 ± 0.022^{c}	0.290 ± 0.013^{*}
Aug.	1.250 ± 0.081^{*d}	4.33 ± 0.34^{*b}	0.240 ± 0.009_{*d}	0.280 ± 0.024^{*}
Sep.	1.050 ± 0.136^{*}	4.16 ± 0.69	0.319 ± 0.104^{*}	0.293 ± 0.068^{*}
Oct.	1.354 ± 0.100^{*}	4.32 ± 0.66^{*}	0.335 ± 0.045^{*}	0.230 ± 0.027
Nov.	1.776 ± 0.162^{*a}	4.57 ± 0.56^{*}	0.436 ± 0.088^{*}	0.253 ± 0.026
Dec.	1.398 ± 0.210^{*}	3.20 ± 0.29^{a}	0.472 ± 0.074^{*}	0.330 ± 0.260^{*a}
Months/ Year 1990				
Jan.	0.928 ± 0.183^{*}	3.73 ± 0.42	0.281 ± 0.095^{*}	0.250 ± 0.020^{*}
Feb.	1.040 ± 0.126^{*}	4.38 ± 0.61^{*}	0.265 ± 0.062^{*}	0.260 ± 0.040
Mar.	0.372 ± 0.067^{b}	4.92 ± 0.55*	0.082 ± 0.019^{a}	0.270 ± 0.040
Apr.	0.736 ± 0.129^{*a}	4.66 ± 0.51^{*}	0.166 ± 0.030^{a}	0.260 ± 0.020

Values were expressed as Mean ± SEM; n, 5n each group.

*Significantly different from the base line value (Smallest mean) July in case T_3 and T_4 ratio; June in case of T_4 and October in case of GSI. Newman – keul's multiple range't' test at a level of 0.05.

a, b, c, d differ from preceding month value at p< 0.05, <0.02, <0.01 and <0.001 respectively (Student's "t" – test).

Table 3: Summary of one-way ANOVA for changes in circulating levels of thyroid hormones during different months of the annual reproductive cycle in male toad, *Bufo melanostictus*

Variables	SS	DF	MS	F	P
Triiodothyronine	15.69	59	-	-	P<0.001
Total					
Between groups	11.51	11	1.05	12.07	
Within groups	4.18	48	0.09	-	
Thyroxine	1343.34	59	-	-	P<0.05
Total					
Between groups	440.00	11	40.01	2.13	
Within groups	903.34	48	18.82	-	
T_3/T_4 ratio	1.67	59	-	-	P<0.001
Total					

Between groups	0.86	11	0.078	4.61	
Within groups	0.81	48	0.017	-	

SS = Sum of square
DF = Degree of freedom
MS = Mean square

Table 4: Circadian changes in thyroid hormone levels in the male toad, *Bufo melanostictus* during pre-spawning phase (late December).

Sampling hrs 1989	T_3 (ng/ml)	T_4 (ng/ml)	T_3/T_4 (ng/ml)
0600	0.218 ± 0.044	5.53 ± 1.09*	0.058 ± 0.030*
0900	0.400 ± 0.032*c	6.90 ± 1.50*	0.078 ± 0.029*
1200	0.155 ± 0.016d	3.07 ± 0.04a	0.051 ± 0.004*
1500	0.890 ± 0.099*d	6.60 ± 1.32*a	0.175 ± 0.059*
1800	0.220 ± 0.035d	7.13 ± 1.08*	0.032 ± 0.003a
2100	0.438 ± 0.103*	5.90 ± 1.10*	0.083 ± 0.027*
2400	0.208 ± 0.030a	3.27 ± 0.42a	0.068 ± 0.013*
0300	0.308 ± 0.059	5.78 ± 1.99*	0.100 ± 0.042*

Values were expressed as Mean ± SEM; n, 5n each group.
*Significantly different from the base line value (smallest mean) 1200 hrs in case of T_3 and T_4 ratio; 1800 hrs in case of T_3/T_4; Newman – keul's multiple range 't' test at a - level of 0.05.
a, b, c, d differ from preceding month value at p< 0.05, <0.02, <0.01 and <0.001 respectively (Student's "t" – test).

Table 5: Circadian changes in thyroid hormone levels in the male toad, *Bufo melanostictus* during spawning phase (late April).

Sampling hrs 1990	T_3 (ng/ml)	T_4 (ng/ml)	T_3/T_4 (ng/ml)
0600	0.305 ± 0.052*	2.95 ± 0.23*	0.103 ± 0.013
0900	0.280 ± 0.018*	2.55 ± 0.24	0.112 ± 0.008
1200	0.318 ± 0.033*	1.93 ± 0.14a	0.172 ± 0.027*
1500	0.288 ± 0.033*	2.40 ± 0.15a	0.122 ± 0.016
1800	0.410 ± 0.084*	2.90 ± 0.19*	0.137 ± 0.028*
2100	0.183 ± 0.051a	1.70 ± 0.16c	0.111 ± 0.038
2400	0.265 ± 0.058*	2.27 ± 0.29	0.120 ± 0.024
0300	0.403 ± 0.079*	2.98 ± 0.63*	0.160 ± 0.054*

Values were expressed as Mean ± SEM; n, 4 in each case.
*Significantly different from the base line value (smallest mean) 2100 hrs in case of T_3 and T_4,0600 hrs in case of T_3/T_4; Newman – keul's multiple range 't' test at a - level of 0.05.
a, b, c, d differ from preceding month value at p< 0.05, <0.02, <0.01 and <0.001 respectively (Student's "t" – test).

Table 6: Circadian changes in thyroid hormone levels in the male toad, *Bufo melanostictus* during preparatory phase (late August).

Sampling hrs 1990	T_3 (ng/ml)	T_4 (ng/ml)	T_3/T_4 (ng/ml)
0600	0.725 ± 0.178*	5.90 ± 0.67*	0.130 ± 0.042
0900	0.397 ± 0.210	4.62 ± 1.34	0.114 ± 0.058
1200	0.405 ± 0.066	6.87 ± 0.91*	0.065 ± 0.016
1500	0.490 ± 0.0128	2.87 ± 0.18d	0.185 ± 0.064
1800	0.560 ± 0.016	6.44 ± 1.20*b	0.102 ± 0.025*
2100	0.340 ± 0.020d	5.00 ± 0.17	0.069 ± 0.007
2400	0.600 ± 0082*b	6.15 ± 1.50*	0.208 ± 0.131
0300	0.340 ± 0.010b	7.20 ± 0.84*	0.051 ± 0.009

Values were expressed as Mean ± SEM; n, 4 in each case.
*Significantly different from the base line value (smallest mean) 2100 hrs and 0300 hrs in case of T_3 and T_4,1500 hrs in case of T_4 and 0300hrs in case of T_3/T_4; Newman – keul's multiple range 't' test at a - level of 0.05.
a, b, c, d differ from preceding month value at p< 0.05, <0.02, <0.01 and <0.001 respectively (Student's "t" – test).

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