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# Synergistic Effect of Melatonin, 5-HTP and L-DOPA on Reproductive Conditions in Spotted Munia *Lonchura Punctulata*

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**Abstract:** *Seasonal changes in reproduction and other metabolic conditions may result from interaction of circadian neural oscillations and hormonal secretion that change seasonally. To test this hypothesis, the present study was designed to study the effect of melatonin and circadian neurotransmitter activity on reproductive and hormonal conditions in a subtropical finch, spotted munia (Lonchura punctulata). During progressive phase of reproductive cycle adult male birds were divided into four groups. Group I administered with normal saline twice a day, group II administered with 5-HTP (serotonin precursor) at 8 AM and L-DOPA (dopamine precursor) at 8 PM, group III received single injection of melatonin in late afternoon. Group IV received melatonin + 5-HTP and L-DOPA. Melatonin and circadian neurotransmitter (Serotonergic and Dopaminergic) activity affected the body weight, testicular growth and plasma LH and testosterone. Birds treated with 5-HTP and L-DOPA showed significantly higher gonadal growth and activity, body weight gain and plasma LH and testosterone level in comparison to control while administration of melatonin alone and together with 5-HTP and L-DOPA induced inhibition but the degree of inhibition in later was less, it seems that melatonin inhibits the full expression of Pituitary-Testicular axis induced by 12-hr circadian phase relation of serotonergic and dopaminergic activity. These findings suggest that seasonal reproductive and hormonal conditions of this avian species might be related to daily rhythm in serotonergic and dopaminergic activities and melatonin secretion; however the exact mechanism by which they drive hypophysial - gonadal axis needs to be ascertained.*

**Keywords:** *Melatonin, Neurotransmitter, Hormones, Testes, Bird.*

## 1. INTRODUCTION

The role of endocrine glands viz. pituitary, adrenal, thyroid and gonadal steroids in the regulation of seasonal reproduction of birds is well established [1, 2]. However, there is scarcity of information on the role of the pineal gland in the regulation of periodic reproduction in the avian species [3]. Melatonin serves as an "internal Zeitgeber" for the brain and body in the vertebrates [4]. Melatonin levels are low during day and high during night, regardless of diurnal or nocturnal habit of animals is well established. Notwithstanding reports on the effects of pinealectomy, there is practically very less information on the effect of melatonin on the annual gonadal development cycles in avian species [3, 5-7]. If there is any role of melatonin in avian reproduction, it appears to be in temporal phasing of the annual reproductive cycle rather than stimulation and termination of gonadal functions [8]. Further, while the mechanism of action of melatonin on the neuroendocrine system of mammals is well established [9, 10], there is complete lack of such information on birds [11]. Unlike in mammals and other non-mammalian vertebrates there is very less information on the time-dependent action of melatonin in birds [3]. The pineal gland of various avian species synthesizes melatonin in a circadian rhythm. Despite of a broad knowledge on molecular and neurochemical mechanisms involved in the control of melatonin generating system in the avian pineal gland, the physiological function (s) of the hormone in this group of animal remains largely unknown.

Pioneer study by Meier and his group suggested the involvement of circadian phase relationship of two hormones i.e., corticosterone and prolactin in the regulation of seasonal events of the photoperiodic migratory bird white throated sparrow, *Zonotrichia albicolis* [12]. This view was further supported when the effects of corticosterone and prolactin administered at different time intervals on a circadian basis could be duplicated by the administration of serotonergic and dopaminergic drugs given on the same ground [13-15]. To induce specific phase relationship between circadian neural oscillators (serotonergic and dopaminergic), 5-HTP (5-Hydroxytryptophan, a precursor of serotonin) and L-DOPA (L-Dihydroxyphenylalanine, a precursor of dopamine) were used because neurotransmitters itself cannot cross the blood brain barrier whereas their precursor do cross [16]. Administration of these precursor drugs at 12 hr interval induced photo-sensitive/breeding condition and at 8 hr interval initiated non-breeding photorefractory conditions. It was later reported in many more avian species that only a 13 days administration of 5-HTP and L-DOPA at an interval of 8-hr was inhibitory and at 12-hr was stimulatory for reproductive conditions [17-20]. It has also been proved that in spotted munia, *Lonchura punctulata* these effects are due to circadian phase relation of serotonergic and dopaminergic activities and not due to serotonin and dopamine alone. Further L-DOPA was effective when converted into dopamine and not into nor-adrenaline or adrenalin, the next biosynthetic product of dopamine [21].

Spotted munia, *Lonchura punctulata* is a seasonally breeding, non- migratory waxbill finch (Family-Estrildidae) distributed all over the Indian sub continent [22]. Adult males and females are similarly pigmented and no sexual dimorphism is apparent. Under natural day length (NDL) seasonal gonad and body weight cycles run parallel to each other [23]. This bird exhibits similar reproductive cycle in nature as well as in captivity but does not follow changes in environmental temperature or / and photoperiod. Since the spotted munia is being used for a number of physiological experiments providing much useful information, it seems therefore, to be an ideal model with which to study the daily interactions, if any between daily and annual rhythm. It has been hypothesized that seasonal changes in reproduction and other metabolic conditions may result from interaction of circadian neural oscillations and hormonal secretion that change seasonally. To test this hypothesis, the present study was designed to study the effect of melatonin and circadian serotonergic and dopaminergic activity in the regulation of seasonal pituitary-gonadal function in spotted munia (*Lonchura punctulata*).

## 2. MATERIALS AND METHODS

60 juvenile spotted munia (First year bird) where purchased from local supplier and kept in indoor aviary. They were provided with natural light and air, food and water *ad libitum*. They were maintained for year. In the second year adult male spotted munia were separated by unilateral laparotomy.

### 2.1. Laparotomy

Birds were anesthetized with sodium pentobarbital and a cut were made with the help of scissor, in the lower abdomen (near last ribs) of left side. Testis was exposed and length and width of the left testis was measured (in mm) with the help of divider and scale. The cut was stitched and necessary post operational cares were made.

### 2.2. Injections

Melatonin (Sigma chemicals, Batch No. M-5250; Lot-77 H 0541, USA) was dissolved in few drops of ethyl alcohol and diluted in normal saline. 5-HTP (5-Hydroxytryptophan, a serotonin precursor- 99% M. W. 220.23; Batch No.10026188, Product No.14168, Lancaster synthesis) and L-DOPA (L-Dihydroxyphenylalanine, a dopamine precursor- M.W. 197.14, Batch No. 01106, and Product No.037079, Central Drug House P. Ltd. Mumbai, India) were dissolved in normal saline with the help of magnetic stirrer.

### 2.3. Doses

Melatonin: 125 ug/100 gm body weight in 0.1ml normal saline/ animal (as described by Murab [25]). 5-HTP and L-DOPA: 5mg /100 gm body weight in 0.1 ml normal saline/ animal (as described by Chaturvedi and Prasad [18]). Normal saline (9%): 0.1 ml/ animal.

### 2.4. Experiments

During progressive phase of reproductive cycle, adult spotted munia (second year bird) were grouped in to 4 wire net cages of six birds each. Before starting the treatment body weight and size of the left testis *in situ* of each bird of all groups were recorded by unilateral laparotomy.

Group I [S: S (saline control)]: Birds received normal saline (0.1ml) twice (i.e. 8 AM and 8 PM) a day.

Group II [5-HTP: L-DOPA (12-hr circadian phase relationship of 5-HTP and L-DOPA)]: Injection of 5-HTP was given at 8 AM and L-DOPA at 8 PM.

Group III [S: Melatonin: S]: Saline were injected as group I and single injection of melatonin was given at late afternoon (05.30 PM).

Group IV [5-HTP: Melatonin: L-DOPA]: 5-HTP and L-DOPA injections were given as Group II and melatonin as Group-III.

All the injections were given intraperitoneally over a period of 45 days. During the treatment period birds were maintained under LL (continuous condition of light) to avoid possible photoperiodic interference due to light: dark cycle with neuroendocrine entrainment by precursor drug injections. Photoperiodic condition was maintained in light proof room fitted with fluorescent light (300 Lux at cage level) and automatic timer. Observations were made before the treatment (Initial value) and then 24 hr after the last injections (at 45 days). Birds were anesthetized and weighed individually. Blood was collected directly from left ventricle of the heart. Blood was centrifuged and plasma with small drop of 0.1% sodium Azide was stored at -20 $^{0c}$ until assayed. Plasma concentrations of LH were measured by micro modification of the method developed by Follett *et al.* [24] and validated for spotted munia plasma [25]. The sensitivity (i.e., 90% B/BO) of the assay was 0·l0 ng/ml (2 pg/tube). Inter- and intra-assay variation was 13·5% ($n$ = 6) and 8·8% ($n$ = 8) respectively. Plasma concentration of testosterone were measured by using method of Boswell [26] and Boswell *et al.* [27] and validated for munia's plasma [25].

The lower limit of detectability of the assay (i.e. the sensitivity of the assay, 90% B/BO) was 0·06 ng/ml (0·12 pg/tube). Inter- and intra-assay variation was 15·4% and 9·2% respectively ($n$ = 8). Reproductive condition was determined by measuring the length and width of the left testis. The volume of testes (in $mm^3$) was calculated by using the formula $4/3\pi ab^2$ (where a = ½ of the long axis and b = ½ of the short axis). Testes were removed and fixed in Bouin's fluid for histological observations. Gonadosomatic index was calculated (mg weight of paired testes/100 gm body weight). During the course of the study the birds were provided with food grains and drinking water *ad libitum*. Experiments on the animals were conducted in accordance with institutional practice and within the framework of revised animals (scientific procedures) Act of 2002 of government of India on animal welfare.

### 2.5. Statistical Analysis

Data were analyzed statistically with the help of student's "t" test, one way analysis of variance (ANOVA) and Newman-Keul's multiple range test (MRT) [28].

## 3. RESULTS

### 3.1. Body Weight and Testicular Volume

Saline treated control group (Group I) of birds experienced normal seasonal increase in the body weight and testicular volume when compared to its initial value.12 hr circadian phase relation of 5-HTP and L-DOPA treated group (Group-II) also experienced increase in body weight and testicular volume but significantly higher than the saline treated control group (Group I) (control vs. 12- hr). Melatonin along with saline treated group (Group-III) showed significantly less value in both body weight and testicular volume when compared to saline treated control group (Group I) and 12-hr circadian phase relation of 5-HTP and L-DOPA treated group (Group-II) (control and 12-hr vs. Melatonin). Group-IV which received melatonin as well as 12-hr circadian phase relation of 5-HTP and L-DOPA also showed significantly lower value of body weight and testicular volume (control and 12-hr vs. 12-hr + Melatonin) but the degree of inhibition is less

than those of melatonin and saline treated birds (Group-III) (Melatonin vs. 12-hr + Melatonin) (Fig.1 and 2). Gonado-somatic index followed the same pattern of result as body weight and testicular volume. (Fig.3)

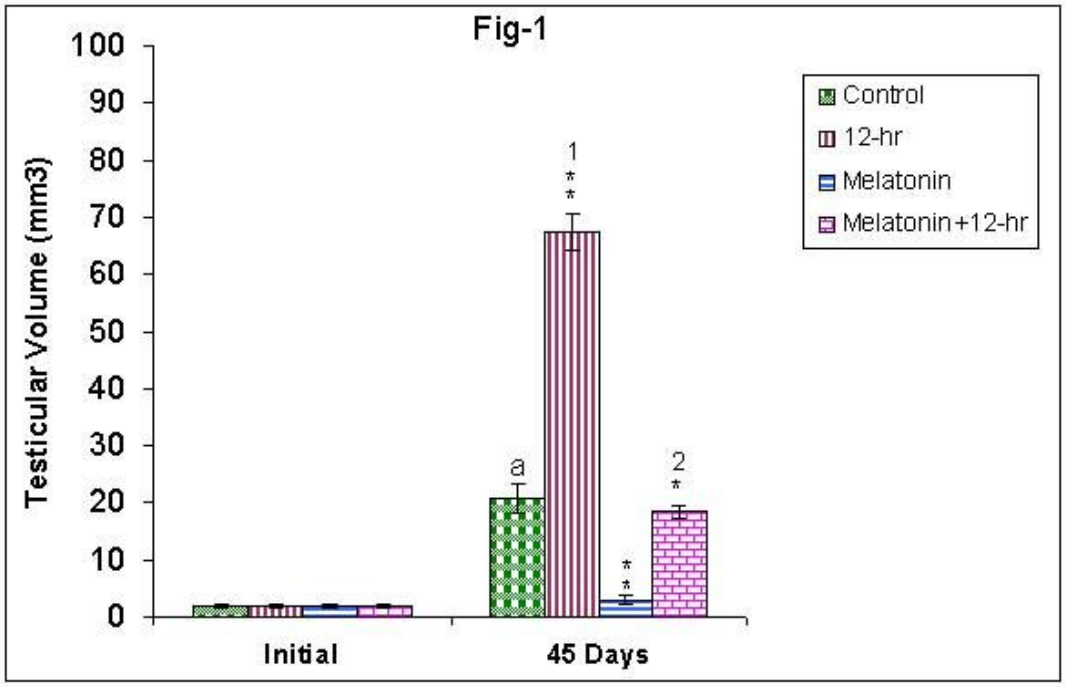


**Fig1.** *Effect of Melatonin, 5-HTP and L-DOPA (12-hr relationship) on the testicular responses of Spotted munia, Lonchura punctulata during progressive phase of reproductive cycle. (Value= Mean ± S.E.). Significance of difference from control (*P<0.05, **P<0.01; student's 't' test).* [a]*Experimental group differ significantly (P<0.001; ANOVA).* [1]*Differ significantly from all other groups (P<0.05; MRT).* [2]*Differ significantly from 12-hr and Melatonin (P<0.05; MRT).*

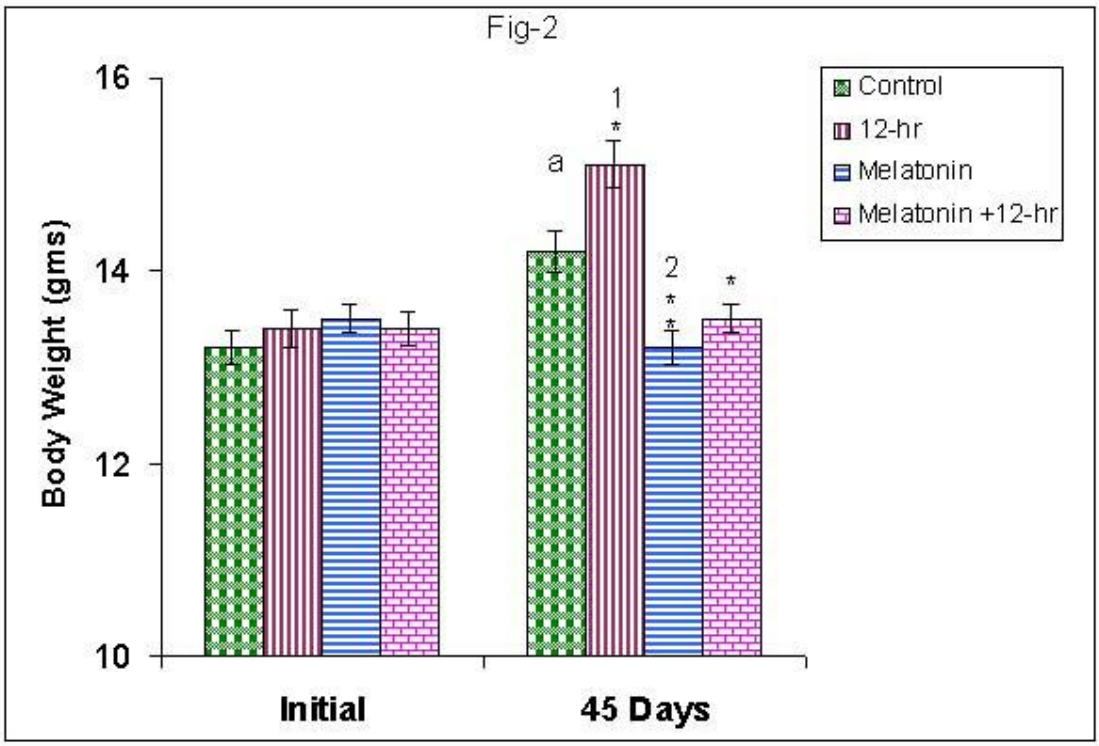


**Fig2**. *Effect of Melatonin, 5-HTP and L-DOPA (12-hr relationship) on the Body weight of Spotted munia, Lonchura punctulata during progressive phase of reproductive cycle. (Value = Mean ± S.E.). Significance of difference from control (*P<0.05, **P<0.01; student's 't' test).* [a]*Experimental group differ significantly (P<0.001; ANOVA).* [1]*Differ significantly from all other groups (P<0.05; MRT).* [2]*Differ significantly from 12-hr and Melatonin (P<0.05; MRT).*

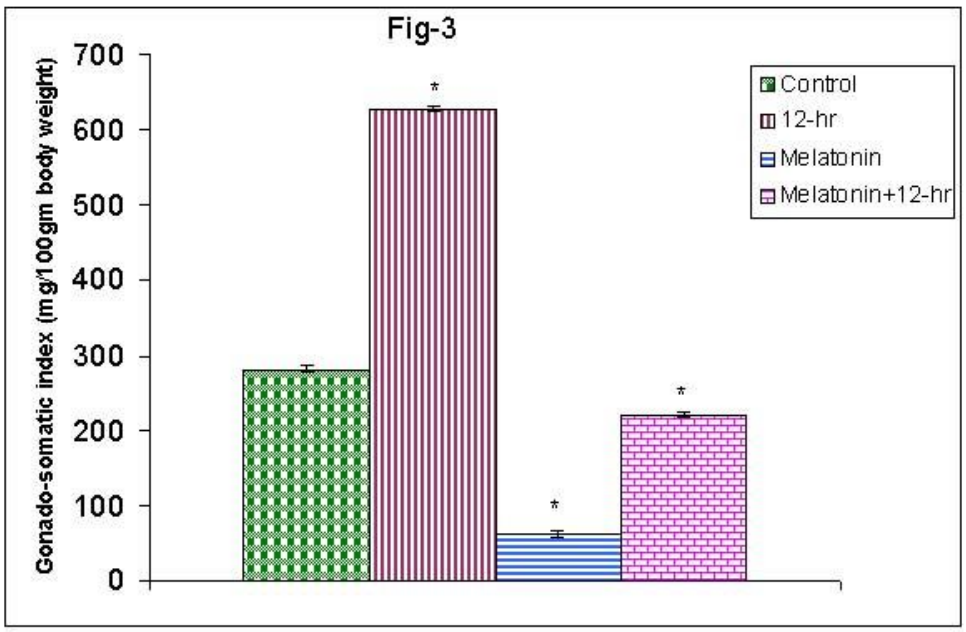


**Fig3.** *Effect of Melatonin, 5-HTP and L-DOPA (12-hr relationship) on the Gonado-somatic index of Spotted munia, Lonchura punctulata during progressive phase of annual reproductive cycle. (Value= Mean ± S.E.). Significance of difference from control (*P<0.001; student's't' test).*

### 3.2. Plasma LH and testosterone

Interestingly, birds treated with 12 hr circadian phase relation of 5-HTP and L-DOPA measured significantly higher level of plasma LH and testosterone in comparison to saline treated control group (control vs. 12-hr). Melatonin with saline treated group showed significantly low plasma LH and testosterone level (control and 12-hr vs. Melatonin). On the other hand the group which was treated with both melatonin and 12 hr circadian phase relation of 5-HTP and L-DOPA together also induced inhibition of plasma LH and testosterone level (control and 12-hr vs. 12-hr + Melatonin) but the degree of inhibition was less than those of melatonin and saline treated birds (Melatonin vs. 12-hr + Melatonin) (Fig.4).

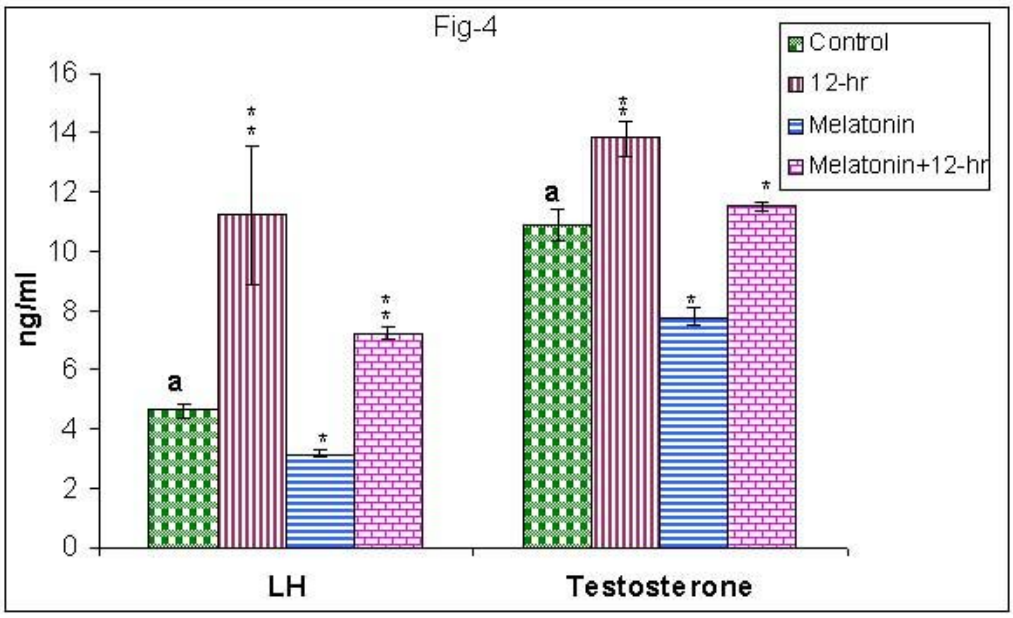


**Fig4.** *Effect of Melatonin, 5-HTP and L-DOPA (12-hr relationship) on Plasma LH and Testosterone level of spotted munia, Lonchura punctulata during progressive phase of reproductive cycle. (Value= Mean ± S.E.). Significance of difference from control (*P<0.05, **P<0.001; student's't' test). [a] Experimental group differ significantly (P<0.001;ANOVA).*

### 3.3. Histological Study of Testes

Histologically, well developed and active seminiferous tubules were found in the testis of both saline treated control and 12 hr circadian phase relation of 5-HTP and L-DOPA treated group [Fig. 5 (1-2)]. They also contained 1-2 layers of spermatogonia, dividing spermatocytes (Primary and secondary), and 2-3 layers of spermatids and bunches of spermatozoa. Leydig cells were not clearly visible. Testis of melatonin with saline treated birds contained small and thick layered seminiferous tubules which had very few or in some totally absent spermatids and spermatozoa. Inactive and aggregated leydig cells were present in the large intertubular spaces [Fig. 5 (3)]. Testes of the group treated with melatonin along with 12 hr circadian phase relation of 5-HTP and L-DOPA also showed inhibited activity as wall of seminiferous tubules was thick and had very few spermatogonia. But in contrast to melatonin with saline treated birds there was dividing spermatocytes and spermatids in the lumen. Leydig cells were not clearly visible [Fig. 5(4)].

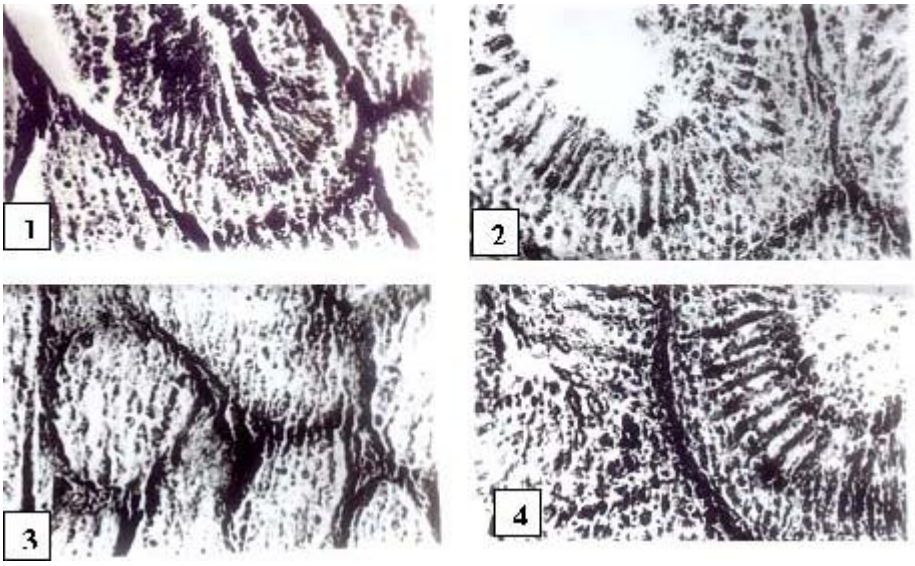


**Fig5.** *Effect of Melatonin, 5-HTP and L-DOPA (12-hr relationship) on the testicular activity of Spotted munia, Lonchura punctulata. (1) T. S. of testis of saline treated control bird×161. (2) T. S. of testis of 12-hr treated bird ×161. (3) T. S. of Testis of Melatonin bird ×161. (4) T .S of testis of Melatonin + 12-hr treated bird ×161.*

## 4. DISCUSSION

Melatonin and circadian neurotransmitter (Serotonergic and Dopaminergic) activity affected the body weight, testicular growth and plasma Luteinizing hormone (LH) and testosterone. Administration of melatonin alone (group III) and together with 5-HTP and L-DOPA (group IV) induced inhibition of seasonal gonadal growth and activity, body weight and plasma LH and testosterone level but the degree of inhibition in later (group IV) was less. On the other hand birds treated with 5-HTP and L-DOPA at 12 hr interval (group II) showed significantly higher gonadal growth and activity, body weight gain and plasma LH and testosterone level in comparison to saline treated control group. Since the degree of gonadal development, body weight gain and increased level of plasma LH and testosterone were less in group IV, it seems that melatonin inhibits the full expression of Pituitary-Testicular axis induced by 12 hr circadian phase relation of serotonergic and dopaminergic activity. Results also demonstrate that late afternoon melatonin administration inhibited body weight and gonadal growth. Plasma LH and testosterone level coincided with the results of body weight and gonadal activity. It is also evident that in spotted munia body weight and gonadal cycle run parallel to each other [23]. Previous report in a non-photoperiodic avian species, Lal munia, *Estrilda amandava*, suggested that melatonin influences gonadal activity and body weight growth by acting directly at the hypothalamo-hypophysial complex [7].

The pineal has an antigonadal activity in the Indian weaver bird, *Ploceus philipinus* [29]. Exogenous melatonin irrespective of its dose and time to injection causes complete inhibition of growth in body weight and LH dependent plumage pigmentation in Lal munia [7]. Ohta and Konishi [30] suggested a role of depressed nocturnal melatonin levels in reproductive activity of

quail. Late afternoon administration of melatonin produces pro-somatotrophic effects in house sparrows [31]. Administration of melatonin for 10 consecutive days causes significant involution of testes during the breeding phase, although it fails to alter reproductive activity during the non-breeding phase in two tropical birds, *Psittacula cyanocephala and Ploceus philippinus* [32]. It has been reported that in male spotted munia, *Lonchura punctulata*, pinealectomy and exogenous melatonin influences thyroid activity and diurnal profiles of blood glucose in relation to reproductive status and time of administration of melatonin [33, 34]. In birds, the pineal organ is photo responsive, contains circadian oscillator(s), and through the production of melatonin provides a daily and seasonal calendar [3]**.** It is also suggested that the avian circadian system may respond to any change in the melatonin rhythm by changing its responsiveness to zeitgeber stimuli [35]. Since the circadian system is involved in regulation of the photoperiodic responses in birds [3, 8, 36], it is logical to expect that melatonin plays a significant role in avian photoperiodism. Melatonin also appears to play a significant role in many physiological functions which are considered non-circadian. For example, in chickens pinealectomy does not affect circadian activity rhythms [37] but induces phase-shifts or disruptions of daily rhythms of certain immune parameters. Rhythmicity could be restored by applications of exogenous melatonin [38]. A similar effect has been recorded in other avian species [3, 39, 40]. Taken together all these studies indicate wide species variability in physiological responses to exogenous melatonin.

Recently Ubuca et al, [41, 42] identified a novel hypothalamic neuropeptide inhibiting gonadotropin release in quail and termed it ganadotropin inhibitory hormone (GnIH).They investigated the mechanisms that regulated GnIH expression and reported that melatonin is a key factor for GnIH induction. Melatonin appears to act directly on GnIH neurons through its receptor to induce GnIH expression. Many bird species are photoperiodic, as are many mammals. Photoperiodic mammals rely on the annual cycle of change on nocturnal secretion of melatonin to drive their reproductive responses [43]**.** In contrast, a dogma has existed that birds do not use seasonal changes in melatonin secretion to time their reproductive effort, and a role for melatonin in birds has remained enigmatic [44, 45]**.** Despite the accepted dogma, there is strong evidence that melatonin is involved in regulation of several seasonal processes, including gonadal activity and gonadotropin secretion [46-48]. In light of these report and considering GnIH's inhibitory effects on gonadotropin secretion [49, 50]. Ubuca et al, [41, 42] hypothesized that melatonin may be involved in the induction of GnIH expression, thus influencing gonadal activity.

The daily injections of the two neurotransmitter precursors (5-HTP and L-DOPA) probably induced a diurnal rhythm in central nervous serotonergic and dopaminergic function [14, 15, 17, 19, 20, 51]. The induction of such putative rhythms results in changes in reproductive function depending on the phase angle between the rhythms suggests that there may be of physiological relevance. A circadian variation in the hypothalamic concentration of neurotransmitters (serotonin and dopamine) has been reported in Japanese quail, *Coturnix coturnix japonica* and suggested that circadian serotonergic and dopaminergic oscillator varies as a function of reproductive status of the bird, and breeding/ non-breeding conditions may be induced experimentally by changing the phase relation of these oscillations [51]. The observation that testicular and body growth can be inhibited by exogenous melatonin (present study) or daily injections of 5-HTP and L-DOPA given at 8-hr interval in spotted munia [18, 21, 52] suggest that a common mechanism could be involved. The converse finding, that seasonal testicular stimulation is prevented by exogenous melatonin and testicular regression is prevented by daily injections of 5-HTP and L-DOPA given 12-hr apart (present study) is consistent with this view. It seems that melatonin counteracts the circadian phase relation of serotonergic and dopaminergic components in the central nervous system and their peripheral expression via hypothalamo-hypophysial-gonadal axis. Results of the present study also suggest that melatonin is more sensitive in progressive phase as it suppress/overcome the stimulatory effect of 5-HTP and L-DOPA given 12-hr apart. These findings also seem to suggest that pineal not only acts as a photo transducer in photosensitive birds [53], but might also be involved in the regulation of the peripheral endocrine glands and reproduction in non-photoperiodic avian species. Further, the physiological actions of melatonin

may vary with the duration, time schedule of the treatment, different phases of the reproductive cycle and from species to species.

It is concluded that (i) melatonin, at least in sub-tropical avian species, exerts an antigonadotrophic effect by acting directly on hypophysial-gonadal axis and (ii) the seasonal changes (breeding and non-breeding) in reproductive and hormonal conditions of this avian species might be related to daily rhythm in serotonergic and dopaminergic activities of the brain and melatonin secreted from the pineal gland, however the exact mechanism by which they drive hypophysial - gonadal axis needs to be ascertained.

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