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# PHOTOPERIODIC REGULATION OF MT1 AND MT2 MELATONIN RECEPTOR EXPRESSION IN SPLEEN AND THYMUS OF A TROPICAL RODENT *FUNAMBULUS PENNANTI* DURING REPRODUCTIVELY ACTIVE AND INACTIVE PHASES

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Photoperiodic regulation of melatonin receptor types on target tissues, such as lymphatic organs, has never been explored for any seasonal breeder. In the present study, we accessed the high affinity membrane melatonin receptors MT1 and MT2 expression dynamics in lymphoid organs (i.e., spleen and thymus) of a seasonally breeding rodent *Funambulus pennanti* during two major reproductive phases (i.e., active and inactive), when the internal hormonal (melatonin and gonadal steroid) as well as the ecological conditions were entirely different. Photoperiod regulates circulatory melatonin level; hence, we noted the effect of different photoperiodic regimes (long; 16L:8D and short; 10L:14D photoperiod) equivalent to summer and winter daylength on membrane melatonin receptor MT1 and MT2 expression in spleen and thymus. We have correlated the melatonin receptor expression with two major hormones varying seasonally (i.e., melatonin and testosterone) also being responsible for modulation of immunity of a seasonal breeder. Differential immunoreactivity of MT1 and MT2 receptor in spleen and thymus of *F. pennanti* suggests an involvement of both the receptor types in signal transduction of photoperiod for seasonal immunomodulation, because in the tropical zone, a slight difference (1:45–2 h) in daylength may change reproductive physiology and immunity of animals for adaptation. Our above suggestion receives strong support from the experiment of photoperiodic exposure on MT1 and MT2 expression at the translational level, where long daylength decreased the circulatory melatonin level and melatonin receptor expression in both lymphatic tissues. On the other hand, under short daylength, expression of MT1 and MT2 receptor increased in both spleen and thymus along with concomitant increase in circulatory melatonin level. Differential hormonal level of melatonin and gonadal hormones during reproductively active and inactive phase and its direct relation with melatonin receptor expression dynamics in lymphoid organs could be

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## INTRODUCTION

Seasonal changes in the immune system are evident in many vertebrate species and are hypothesized to have evolved to facilitate survival in terms of annual cycles in pathogen prevalence and energy availability (Nelson et al., 1995, 1998). The annual change in photoperiod is the most reliable proximate cue that predicts seasonal challenges in climate, nutrition, and opportunistic pathogens. Substantial research demonstrates that changes in daylength induce not only changes in reproduction, but also changes in immunity (Demas & Nelson, 2003; Nelson & Demas, 1996).

Melatonin, a hormone derived from the pineal gland, is a biological signal of daylength and is a well-established modulator of immunity (Maestroni et al., 1986; Singh & Haldar, 2007). It has been proposed elsewhere that melatonin plays a pivotal role in seasonal adjustments of immunity in tropical rodents (Rai & Haldar, 2003). Earlier, results suggested that the significance of annual variation in reproduction as well as level of melatonin could also be responsible for changes in immune status of various seasonally breeding mammals of temperate and tropical zones (Nelson & Demas, 1996; Rai & Haldar, 2003), although in the tropical zone, the differences in summer and winter daylength are much less (1:45–2 h). It is now a well-known fact that the short-day condition increases the duration of melatonin secretion, which in turn induces immunity in most vertebrates, including humans (Pandi-Perumal et al., 2006). Specific membrane and nuclear binding sites for melatonin have been described in many different immune tissues of different mammalian species, including humans. Over the last few years, there have been great advances in molecular studies on melatonin receptors, and two mammalian melatonin membrane receptors have been cloned (i.e., MT1 and MT2) (Dubocovich & Markiwska, 2005; Reppert et al., 1994, 1995). Despite the presence of melatonin receptors in a wide variety of tissues, reproductive phase-dependent variation and regulation of the membrane receptors on the immune system by photoperiod in any rodent have not been studied in detail. Thus, we are reporting the expression dynamics of melatonin membrane receptor types (MT1 and MT2) in the thymus and spleen of the Indian tropical palm squirrel following different photoperiodic exposures (control conditions of 12L:12D, short-day conditions of 10L:14D, and long-day conditions of 16L:8D) during two reproductive (inactive and active) phases occurring under two different seasons in

nature when the internal hormonal level of melatonin and gonadal steroid is entirely different. We also propose that variation in the circulatory level of melatonin and expression of melatonin receptor types on lymphatic tissue is responsible for providing immunological protection to the animals and is primarily controlled by the variation in photoperiod, as evidenced by its slight changes in the tropics.

## MATERIAL AND METHODS

### Animal Maintenance

Indian Palm squirrel *Funambulus pennanti* is a seasonal (long-day) breeder of Indian origin. It is semi-domestic in nature. Details of its habit, habitat, and reproductive pattern have been published elsewhere (Haldar & Saxena, 1988). Adult male squirrels of average weight 100 ± 10 g (mean ± SE) were collected from the vicinity of Varanasi (latitude 25°, 18' N; longitude 83°, 1'E) and acclimatized to laboratory conditions for one week in a room fully exposed to ambient conditions. They were kept in groups of seven in wire-net cages (25" × 25" × 30" in size) during experiments and were maintained in a well-ventilated room exposed to ambient conditions. Squirrels were fed with soaked gram seeds (*Cicer arientium*), nuts, and seasonal fruits/vegetables and provided water ad libitum. All the experiments on the animals were conducted in accordance with institutional practice and within the framework of the revised Animal (Specific Procedure) Act of 2007 of Govt. of India on animal welfare and were also in line with the ethical standards of the journal (Portaluppi et al., 2008).

### Photoperiodic Treatment/Exposure

Group 1 consisted of randomly selected seven adult male squirrels (body weight 100 ± 10 g range) that were exposed to a light condition equivalent to natural daylength of the tropical zone (12L:12D; lights-on at 08:00 h and lights-off at 20:00 h) and were treated as controls. Group 2 consisted of another seven male squirrels exposed to short-daylength conditions (10L:14D; lights-on at 10:00 h and lights-off at 20:00 h). Group 3 consisted of seven male squirrels exposed to long-day photoperiodic conditions (16L:08D; lights-on at 4:00 h and lights-off at 20:00 h). Squirrels were maintained in the respective conditions for 10 weeks. At the end of each experiment, the animals were weighed and sacrificed at 22:00 h (when both the photoperiodic Groups 2 and 3 along with control squirrels experienced the dark phase) by decapitation. Spleen and thymus were processed for western blot analysis, while trunk blood was collected in a heparinized tube. Plasma was separated and stored at –20°C until RIA analysis of hormones.

Apart from the photoperiodic experiment, we also studied seven squirrels to gather seasonal control data. Animals were sacrificed during both the reproductively inactive (winter: November–December) and active (summer: May–June) phases, after a week of acclimatization to ambient conditions in the laboratory without any treatment. Spleen and thymus were collected and processed for immunoblot analysis, and plasma was stored at –20°C for radioimmunoassay.

### Validation of Melatonin (MT1 and MT2) Receptor Antibody

MT1 R and MT2 R antibody was purchased from Santa Cruz, Biotech (Mel1aR; R-18, sc-13186 & Mel1bR; T-18, sc-13177, Santa Cruz Biotech, Santa Cruz, California, USA). As these antibodies were used for the first time for this rodent, their specificity had to be checked and validated in target spleen and thymus tissue. For validation of antibodies of the MT1 and MT2 receptors, western blot was performed by comparing MT1 and MT2 immunoreactivity in squirrel spleen, thymus, and brain tissue with positive control (i.e., rat brain) (Figures 1c and 1e). A pre-stained multicolor broad range marker (Spectra$^{TM}$ multicolor broad range marker; 10-260 kDa #SM-1841; Fermentas, Glen Burnie, Maryland, USA) was also run along with sample proteins to clarify the position of the band obtained. Western blot analysis for both MT1 and MT2 in spleen, thymus, and brain tissue of squirrel showed a single band between 35–40 kDa, corresponding to similar band obtained for rat brain, which was used as positive control. Validation of Western blot assay for MT1 and MT2 receptor protein was further confirmed using serially diluted protein samples ranging between 10 and 140 μg (Figures 2c and 2d). The intensity of the protein bands of the Western blot for MT1 and MT2 receptor protein was quantified using densitometry software (Scion Image software, Frederick, Maryland, USA). The plotted graph relating the amount of protein loaded and intensity of the protein bands showed strong correlation in spleen tissue protein ($r = 0.87$ for MT1 and $r = 0.85$ for MT2) (Figures 2c and 2d).

### Immunohistochemical Localization of Melatonin Receptor Types (MT1 and MT2)

For immunohistochemical localization of melatonin receptor types (MT1 and MT2), spleen and thymus were dissected out and fixed in Bouin's fluid for 24 h by immersion. After dehydration, paraffin blocks were prepared and 7 μm-thick sections were cut and mounted on gelatin (1%) coated slides and deparafinized. After rehydration, endogenous peroxide activity was blocked by 0.3% $H_2O_2$ in methanol for 30 min at room temperature. Sections were washed thrice with phosphate buffer saline (PBS; 0.1 M $NaH_2PO_4$, $Na_2HPO_4$, NaCl; pH 7.4) and preincubated with

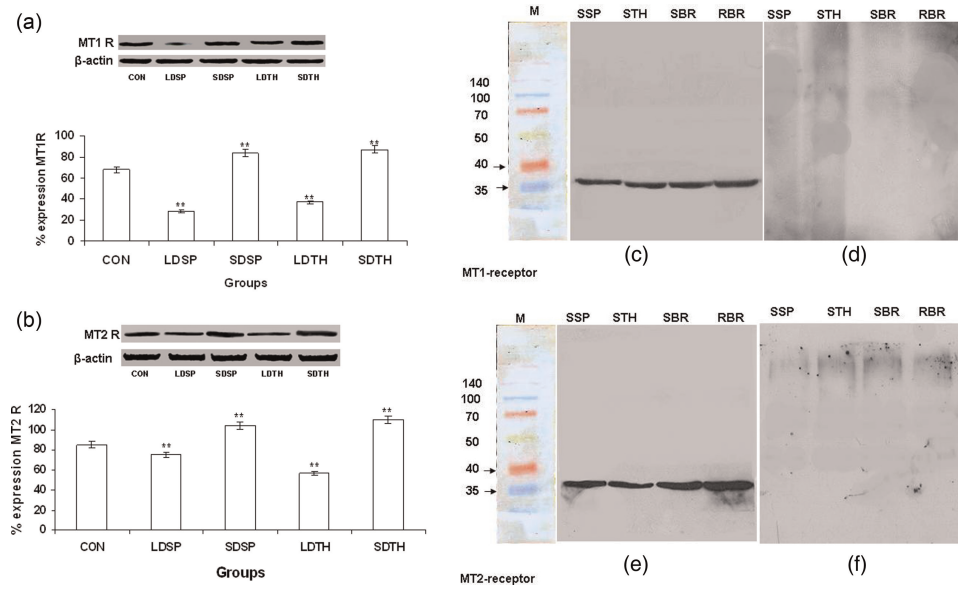


**FIGURE 1** Western blot analysis of (a) MT1 and (b) MT2 receptor expression in spleen and thymus of *F. pennanti* under different photoperiodic regimes. β-actin expression was used as a loading control. Lower panel shows the percent expression of receptor following Scion image analysis. Histogram represent mean ± SEM, n = 4. Control vs. experimental significance of difference $^{**}p < 0.01$. Western blot of (c) MT1 and (e) MT2 receptor antiserum in target tissues with positive control showing single major specific band between 35–40 kDa compared with standard marker protein (M), which has been run along with protein samples and scanned after transferring onto nitrocellulose membrane from the gel. Control western blot of (d) MT1 and (f) MT2 receptor antiserum in target tissues preabsorbed with their respective antigenic peptides showing no specific band. Abbreviations: CON = control, LDSP = long-day spleen, LDTH = long-day thymus, SDTH = short-day thymus, SSP = squirrel spleen, STH = squirrel thymus, SBR = squirrel brain, RBR = rat brain, M-Pre = stained standard marker protein. Photoperiods abbreviations: CON = 12L:12D, long-day = 16L:8D, short-day = 10L:14D.

horse blocking serum (1:100 in PBS; PK-6200, Vector Laboratories, Burlingame, CA) for 2 h. Sections were incubated with primary antibody (Mel1aR; R-18, dilution 1:200 & Mel1bR; T-18, dilution 1:200, Santa Cruz Biotech) overnight at 4°C. Sections were washed thrice with PBS and then incubated with biotinylated secondary antibody (Vectastain ABC Universal kit; PK-6200, dilution 1:50, Vector Laboratories). After washing with PBS, a pre-formed ABC reagent was added conjugated to the free biotin of the secondary antibody. The antigens were visualized using the 0.03% peroxidase substrate 3, 3-diaminobenzidine (DAB, Sigma Chemicals, St. Louis, Missouri, USA) in 0.01M Tris-Cl (pH 7.6) and 0.1% H2O2 and counterstained with Ehrlich's hematoxylin (Savaskan et al., 2002). To test the specificity of the used antibodies, a preabsorption method was used for both the spleen and thymus tissues. The primary antibodies in the same dilution as used for localization (i.e., 1:200) were replaced by a preabsorbed mixture of MT1 and MT2 receptor antiserum

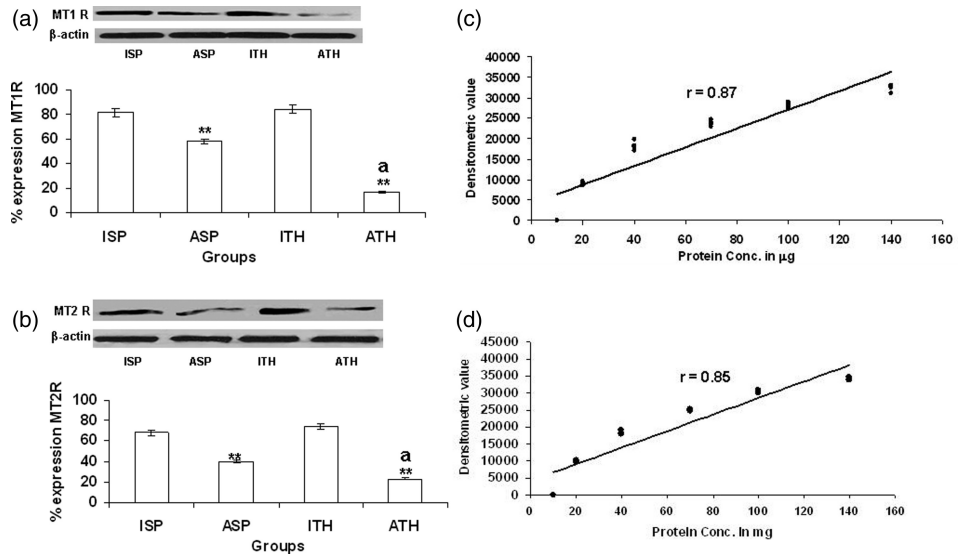


**FIGURE 2** Western blot analysis of (a) MT1 and (b) MT2 receptor expression in spleen and thymus of *F. pennanti* during the reproductively inactive and active phases. β-actin expression was used as a loading control. Lower panel shows the percent expression of receptor following Scion image analysis. Histogram represent mean ± SEM, n = 4. Control vs. experimental significant difference $^{**}p < 0.01$; ITH vs. ATH significant difference, $^{a}p < 0.01$. Validation curve for western blot in spleen depicting amount of protein loaded and intensity of band obtained, showing strong correlation for (c) MT1 receptor (r = 0.87) and (d) MT2 receptor, respectively (r = 0.85). Abbreviations: ISP = inactive spleen, ITH = inactive thymus, ASP = active spleen, ATH = active thymus, reproductively inactive = RIP, reproductively active = RAP.

and their respective antigenic peptide (600 ng/100 µl of MT1 receptor; sc-13186P and 600 ng/100 µl of MT2 receptor; sc-13177P peptides, Santa Cruz Biotech). For preabsorption, the antigens were added to the same diluted antisera (MT1 and MT2 receptor; 1:200), incubated overnight at 4°C, and centrifuged, and then the supernatant was used. Finally, sections were observed and photographed under Leitz-MPV-3 microscope (Germany) and documented.

### Western Blot for Melatonin Receptor Types (MT1 and MT2)

Spleen and thymus were homogenized and lysed in lysis buffer (RIPA buffer containing aprotinin, sodium orthovanadate, and phenylmethylsulfonylfluoride [PMSF]) and quantified by the Bradford (1976) method. Aliquots containing 70 µg protein for melatonin receptor types (Mel1aR or MT1 R and Mel1bR or MT2 R) were resolved with 12% SDS-polyacrylamide gel electrophoresis and followed by electrotransfer (Biometra, Goettingen, Germany) to nitrocellulose membrane (Bioscience, Keene, New Hampshire, USA) for 1 h. The nitrocellulose membrane was blocked

for 60 min. with Tris-buffered saline (TBS; Tris 50mM (pH 7.5), NaCl 150mM) containing 5% fat-free dry milk, and incubated with melatonin receptor antibodies (Mel1aR, R-18 and Mel1bR, T-18 in 1:200 dilution, Santa Cruz Biotech). Membranes were then washed with three changes of TBS over 10 min. Immunodetection was performed with horseradish peroxidase conjugated secondary antibody (donkey anti-goat IgG-HRP diluted 1:1000). Finally, the blot was washed three times with TBS and developed by with Super Signal West Pico Chemiluminescent Substrate (#34080, Thermo Scientific, Rockford, Maryland, USA). Similarly, the blot was developed for β-actin (A-2228, Sigma-Aldrich Chemicals) in 1:1000 dilution as a loading control. Immunodetection of β-actin was performed with anti-mouse IgG-HRP (1:1000). Bands were quantified by measurement of optical density using Scion Image Analysis Software (Scion Corporation). Values were expressed as the ratio of the density of the specific signal to the β-actin signal (Chattoraj et al., 2009; Treeck et al., 2006). Data of four different experiments were pooled. To test the specificity of the antibody, primary antiserum in the same dilution (MT1 R and MT2 R, 1:200 dilution) were preabsorbed with their respective pure antigenic peptide in the concentration of 600 ng/100 µl (MT1 R; sc-13186P and MT2 R; sc-13177P peptides, Santa Cruz Biotech) and incubated overnight at 4°C. With this preabsorbed antiserum, western blot was performed in all the target tissues in the same way as explained above.

### Radioimmunoassay (RIA)

The radioimmunoassay (RIA) of melatonin was performed following the method of Rollag and Niswender (1976) using Guildhey antisera (Guildhey, Surrey, UK). Details of the method can be found elsewhere (Dubey & Haldar, 1997). The validation of the radioimmunoassay was performed as described earlier (Dubey & Haldar, 1997). The intra- and inter-assay variation was 9 and 15%, respectively. The sensitivity for melatonin RIA was 18–20 pg/ml and recovery was 92%. Radioimmunoassay of testosterone was performed according to the manufacturer's instruction (Immunotech, France). The intra- and inter-assay variation was 14.8% and 15%, respectively. The sensitivity was between 0.025–20 ng/ml, and the recovery percentage was between 91 and 117.

### Statistical Analysis

Statistical analysis of the data was performed by one-way ANOVA followed by the Student Newman–Keuls multiple range test. The differences were considered significant when $p < 0.05$. Correlation analyses were also performed.

## RESULTS

### Immunohistochemical Analysis

Strong immunoreactivity for the MT1 receptor was observed on the membranes of splenocytes and thymocytes. Strong immunoreactivity was observed in different regions of the spleen and thymus when compared to the MT2 receptor (Figures 3 and 4). Thymus showed more immunopositive cells for both receptor antigens than spleen. Thymus showed immunoreactivity for both melatonin receptor types (MT1 and MT2) in the cortical and medullary areas. Thymocytes in the cortex and Hassall's corpuscles in the medulla region showed strong immunoreactivity (Figure 4). In spleen, the white pulp showed strong immunoreactivity for splenic lymphocytes (splenocytes), blood vessels, and trabeculae (Figure 3). No immunoreactivity was observed in sections of the spleen and thymus incubated with preabsorbed antiserum of MT1 and MT2 receptors, respectively (Figures 3g, 3h, 4g, and 4h).

### Western Blot Analysis

We performed western blot analysis to assess the expression of melatonin receptor types in the lymphoid tissues of the spleen and thymus of *F. pennanti* at the translational level. Both melatonin receptor (MT1 R and MT2 R) proteins as a single band in between 35–40 kDa were detected in both spleen and thymus, which precisely corresponded to the predicted molecular mass of the receptor (Chattoraj et al., 2009; Dubocovich et al., 2003; Song et al., 1997).

In both spleen and thymus, both MT1 and MT2 receptors showed significant decrease ($p < 0.01$) in percent expression during the reproductively active phase compared to the reproductively inactive phase. Thymus as compared to spleen showed greater decrease in the expression of both MT1 and MT2 receptors during the reproductively active phase than reproductively inactive phase (Figures 2a and 2b). Percent expression for both MT1 and MT2 receptors was higher in thymus than spleen.

The MT1 receptor in the thymus showed significant increase ($p < 0.01$) in percent expression following the short-day (10L:14D) condition, while a significant decrease was noted during the long-day condition when compared with control squirrels kept under 12L:12D. Similarly, the spleen MT1 receptor showed a significant decrease ($p < 0.01$) in percent expression under the long-day condition compared with squirrels kept under the control 12L:12D exposure, but a significant increase ($p < 0.01$) compared to animals kept under the short-day condition when compared to the control 12L:12D exposure (Figure 1a).

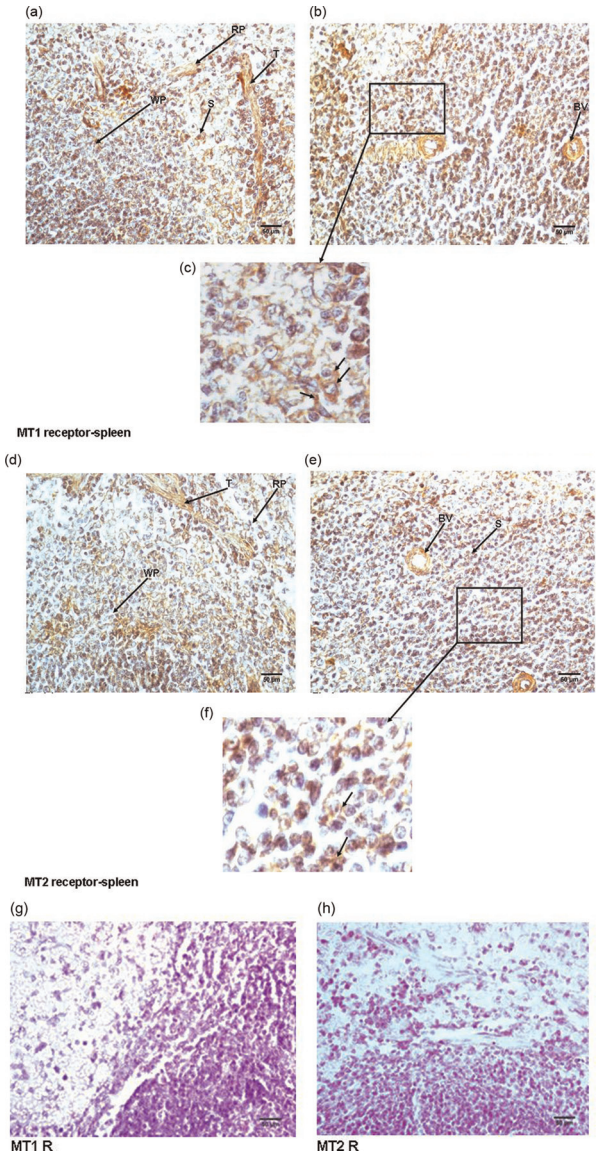


**FIGURE 3** Immunolocalization of the MT1 and MT2 receptors in the spleen of *F. pennanti*. Intense immunoreactivity was noted in spleenocytes near the white pulp and mild immunoreactivity in red pulp area. Intense immunostaining against the MT1 receptor (a–c) was noted when compared to the MT2 receptor (d–f) in different regions of the spleen. Preabsorbed control sections of spleen for (g) MT1 and (h) MT2 receptors showing no immunoreactivity. Note the immunoreactivity against (c) MT1 and (f) MT2 receptors on membranes of splenocytes. Black arrows = immunoreactive cells. Abbreviations: S = splenocytes, WP = white pulp, RP = red pulp, BV = blood vessels, T = trabeculae.

The MT2 receptor in spleen showed a significant decrease ($p < 0.01$) in percent expression under the long-day condition, while it showed a significant increase ($p < 0.01$) in percent expression under the short-day

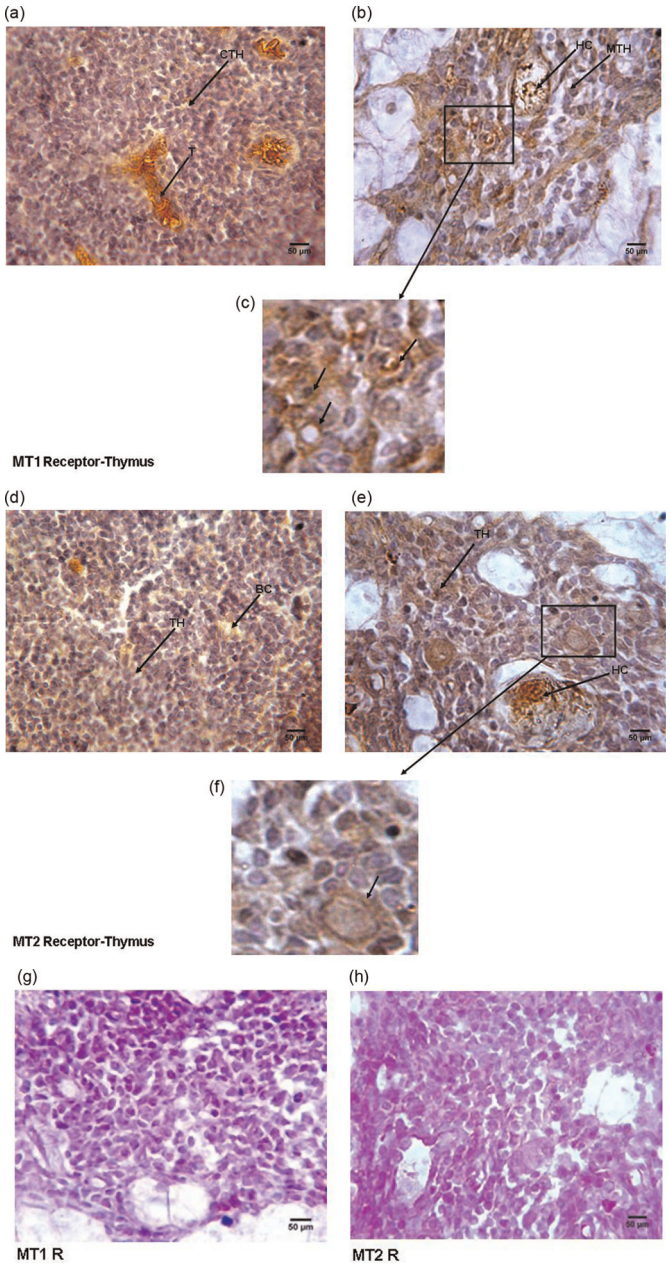


**FIGURE 4** Immunolocalization of the MT1 (a–c) and MT2 (d–f) receptor in the different areas of thymus of *F. pennanti*. Note the intense immunoreactivity in (b, e) medullary region (medullary thymocytes, Hassal's corpuscles, and endothelial blood vessels) as compared to (a, d) cortical region. Preabsorbed control sections of thymus for (g) MT1 and (h) MT2 receptor showing no immunoreactivity. Black arrows = immunoreactive cells. Note the immunoreactivity against the (c) MT1 and (f) MT2 receptor on membranes of thymocytes. Abbreviations: CTH = cortical thymocytes, MTH = medullary thymocytes, HC = Hassall's corpuscles, BV = blood vessels.

condition compared with the control 12L:12D exposure. Similarly, in the thymus, there was a significant decrease ($p < 0.01$) under the long-day condition and a significant increase ($p < 0.01$) under the short-day condition in the MT2 receptor percent expression compared with the control 12L:12D exposure (Figure 1b). The MT2 receptor showed a higher percent expression than the MT1 receptor in almost all photoperiodic groups. No specific band was found in the blot processed with MT1 and MT2 receptor antibody preabsorbed with their respective antigen (Figures 1d and 1f), while a single major band was expressed in both the spleen and thymus between 35–40 kDa, similar to the positive control (i.e., rat brain tissue). No signal was observed in the preabsorbed protein samples even after overexposure (5 min) using the ECL method.

## Radioimmunoassay (RIA)

The circulatory level of melatonin was significantly higher ($p < 0.01$) during the reproductively inactive than active phase (Figure 5a). The circulatory level of testosterone was also significantly higher ($p < 0.01$) during the reproductively active than reproductively inactive phase (Figure 5b). When squirrels were exposed to the different photoperiodic

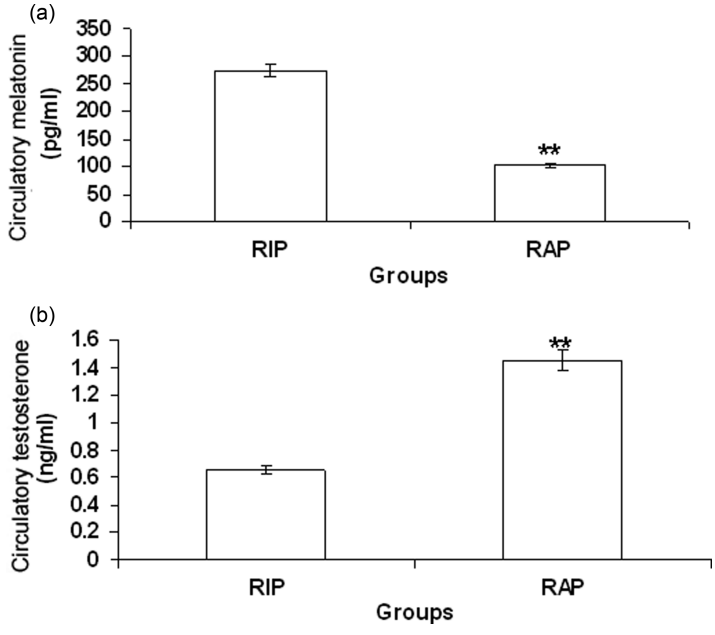


**FIGURE 5** Effect of different reproductive phases (inactive and active) on (a) circulatory melatonin (pg/ml) and (b) testosterone (ng/ml) levels of the Indian palm squirrel *F. pennanti*. Values are mean ± SEM; n = 7. Abbreviations: RIP = reproductively inactive phase vs. RAP = reproductively active phase significance of difference, $^{**}p < 0.01$.

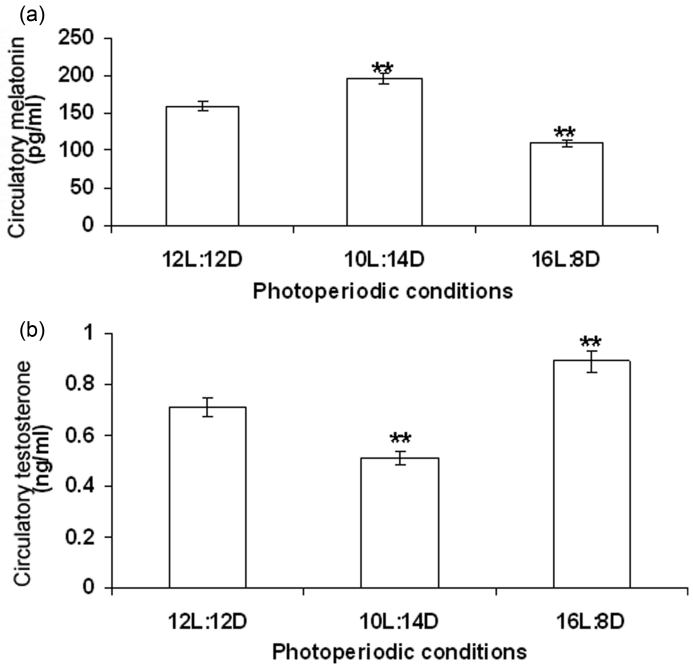


**FIGURE 6** Effect of different photoperiodic regimes (control = 12L:12D, long-day = 16L:8D, and short-day = 10L:14D) on (a) circulatory melatonin (pg/ml) and (b) testosterone (ng/ml) level of the Indian palm squirrel *F. pennanti*. Values are mean ± SEM; n = 7. Control vs. photoperiodic groups significance of difference, $^{**}p < 0.01$.

schedules, the circulatory level of melatonin was significantly elevated ($p < 0.01$) under the short-day condition but significantly reduced ($p < 0.01$) under the long-day condition compared with 12L:12D exposure (Figure 6a). Long-day exposed squirrels showed a significantly ($p < 0.01$) elevated level of plasma testosterone, while short-day exposed squirrels showed a significantly ($p < 0.01$) reduced level when compared with control squirrels kept under 12L:12D (Figure 6b).

## DISCUSSION

The Indian palm squirrel *F. pennanti* is a photoresponsive tropical seasonal breeder presenting two different major (active and inactive) reproductive phases during the year (Haldar et al., 2001) to adjust its various physiological functions along with immunity and reproduction (Ahmad & Haldar, 2009; Haldar & Saxena, 1988). Melatonin is the major hormone involved in maintaining seasonality in this rodent (Haldar & Singh, 2001; Haldar et al., 2001). Further, the duration and concentration of melatonin secretion varies according to the photoperiodic conditions, and this helps to keep the animals healthy and protected (Maestroni et al., 1986; Nelson

et al., 1995, 1998). The question of whether the L/D schedule, which influences the level of melatonin, can affect melatonin receptor expression in target tissues responsible for immune function has never been explored, and, hence, it was accessed in the present study.

In mammals, the thymus is known as a primary and the spleen as a secondary lymphoid organ. Especially in Indian palm squirrels, the thymus is found active in the adult stage (Haldar & Singh, 2001); hence, the role of this lymphoid organ in modulating immunity could be of high adaptive significance for a tropical seasonal breeder. We therefore localized melatonin receptor types in the thymus and spleen and found a strong immunoreactivity against the MT1 receptor on thymocytes, rather than splenocytes, while both lymphoid organs presented immunoreactivity for both the melatonin receptors. The MT1 receptor showed high immunoreactivity in comparison to the MT2 receptor in spleen; hence, a tissue-specific melatonin receptor expression was noted.

High-affinity melatonin receptors have been localized on circulating lymphocytes of rodents, chickens, and humans (Calvo et al., 1995; Pang & Pang, 1992; Pang et al., 1995) and also on thymocytes and splenocytes of humans, several rodents, and bird species (Lopez Gonzales et al., 1993; Martin-Cacao et al., 1993; Rafii-El-Idrissi et al., 1995). Until now, no report suggested photoperiodic regulation of melatonin receptors in lymphoid organs of any tropical seasonal breeder, in general, and especially the Indian palm squirrel, where not only the spleen but the thymus plays an important role in maintaining immune function according to the seasons (Ahmad & Haldar, 2009; Haldar & Singh, 2001; Haldar et al., 2001). This seasonal adjustment of immune function of *F. pennanti* is regulated by daylength and the duration of melatonin in the circulation. Therefore, we observed a high circulatory level and a longer duration of melatonin secretion during the reproductively inactive phase and a significantly low level of melatonin of shorter duration of secretion during the reproductively active phase (Haldar et al., 2004). The spleen and thymus both showed variation in their response as judged by their mass and the blastogenic response of splenocytes and thymocytes during the reproductively active and inactive phases (Ahmad & Haldar, 2009).

We therefore assessed the percent expression of the high affinity melatonin MT1 and MT2 receptors during the two reproductive phases: the reproductively inactive one, which occurs in the winter months (i.e., November–December) and corresponds to the experimental short-day with longer duration of melatonin, and the reproductively active one, which occurs in the summer months (May–June) and corresponds to the experimental long-days and shorter duration of melatonin. Interestingly, a reproductive phase-dependent expression of both MT1 and MT2 receptors in the lymphoid tissues of both the spleen and thymus was

noted; expression was high during the reproductively inactive phase but low during the reproductively active phase. This differential dynamics of the expression of the melatonin receptors in the spleen and thymus might reflect a definite role in maintaining optimal status of health during different reproductive phases occurring in summer and winter.

The high level of circulatory melatonin with its extended duration of excretion complemented the elevated expression of the melatonin receptor types during the reproductively inactive phase. Similarly, the low expression of both melatonin receptor types during the reproductively inactive phase parallels the low level of circulatory melatonin and its short duration of excretion. Previous studies on melatonin receptor expression in the suprachiasmatic nucleus of rat suggest that a high melatonin level coincides with elevated expression of melatonin receptors (Gerdin et al., 2004; Laitinen et al., 1989). Furthermore, a high melatonin concentration during the reproductively inactive phase along with high expression of melatonin receptors maintained high immune status in winter—a stressful condition for tropical mammals—and an opposite relation during the reproductively active phase could be beneficial to immunity.

To check this suggestion, we extended our study by exposing the squirrels to different photoperiodic condition. We imposed the 12L:12D exposure as control, because most of the year squirrels experience ~12 h light in the tropical zone. Further, to overcome the problem of the photoperiodic transitional changes in nature (summer to winter and vice versa), we selected the 12L:12D condition as the control photoperiod, while all the other ecofactors (i.e., temperature and relative humidity) remained the same as in the ambient conditions during the reproductively inactive and active phases. The winter photoperiod noted so far in the region of Varanasi is ~10 h of light, so the short photoperiod selected had a duration of 10L:14D. The longest photoperiod experienced by squirrel in Varanasi is ~14 h, so we exposed them to a little bit longer photoperiod of 16L:8D duration.

Our immunoblot analysis for both the MT1 and MT2 melatonin receptor types in both lymphoid tissues showed increased expression under the short-day photoperiod and decreased expression under the long-day photoperiod compared with the control 12L:12D condition. The MT1 receptor showed major change in its expression pattern during the short-day and long-day conditions in both the spleen and thymus compared with MT2 receptor expression, thus suggesting a prominent role for the MT1 receptor in the photoperiodic regulation of immunity. A recent report on mice suggests that both MT1 and MT2 receptors are possibly involved in photoperiodic regulation of reproductive function (Yasuo et al., 2009). Further, the role of MT2 receptor expression on lymphoid tissue should not be neglected, as we also noted important changes in its expression pattern.

Androgens can be important mediators of seasonal adjustment of immunity with reproduction in a seasonal breeder (Ahmad & Haldar, 2009; Nelson & Demas, 1996; Rai & Haldar, 2003). A trade-off relationship between melatonin and testosterone was reported for Indian palm squirrels (Ahmad & Haldar, 2009), though both hormones act in concert to provide the best possible and optimal immune condition for the survival of squirrels in relation to reproduction under the different seasonal conditions. When we checked the testosterone level in different photoperiodically exposed groups, we found a significantly reduced level in short-day (10L:14D) condition corresponding to the reproductively inactive phase, while we found a significantly elevated level in long-day (16L:8D) condition corresponding to the reproductively active phase when compared with control group (12L:12D). Thus, during the reproductively active period (summer long-day condition of 16L:8D), the decreased level of melatonin and reduced expression of melatonin receptor types coincided with the high circulatory level of testosterone. Furthermore, the increased melatonin level along with high melatonin receptor MT1 and MT2 type expression coincided with the decreased level of testosterone during the reproductively inactive period (winter short-day condition of 10L:14D). Many cells adjust the number of receptors they express according to the signals that activates them (Goodman, 2009). Cells can adjust the abundance of their hormone receptor and hence their responsiveness toward hormone changes according to changing physiological circumstances (Gether, 2000). In our case, the hormone is melatonin and the physiological circumstances are the responses to the changing photoperiod. It is also evident that the pattern, rather than amount, of hormone secretion is of great importance in determining hormone receptor responses (Dannies, 1999). Thus, a small change in melatonin level caused by slight change in tropical photoperiod might have changed the receptor dynamics. Such a type of hormonal interplay and its intricate relation with the expression of the MT1 and MT2 melatonin receptor type might be acting as a signal in mediating seasonal adjustment of two of the most important mega-physiological events (i.e., immunity and reproduction), required for the survival of the organism. Therefore, it may be hypothesized that the changing photoperiodic condition caused alteration not only of the circulatory level of melatonin but also the expression pattern of the MT1 and MT2 melatonin receptor types in the lymphoid organs of the spleen and thymus in the tropical seasonally breeding rodent *F. pennanti*. However, more study is required to answer specifically the questions as to which membrane the melatonin receptor is involved in modulating the immunity of the tropical rodents, and our laboratory is working in that direction.

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