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ORIGINAL ARTICLE

Photoperiod–Testicular–Immune Interaction in a Seasonal Breeder Indian Palm Squirrel *Funambulus pennanti* During the Reproductively Inactive and Active Phases

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The differential effect of long (LD; 16 : 8 h light/dark), short (SD; 10 : 14 h light/dark) and natural day length (NDL; 12 : 12 h light/dark) during the reproductively inactive (RIP) and active (RAP) phases was assessed in relation to immunity and reproductive function of a tropical rodent *Funambulus pennanti*. They presented high immunity and low testicular activity during RIP and an opposite during RAP. SD increased spleen and thymus weight, leukocyte and lymphocyte counts, cell mediated immunity [i.e. blastogenic response in terms of percentage stimulation ratio of splenocytes and thymocytes (when challenged with concanavalin A)] and delayed type hypersensitivity to oxazolone. SD during RIP increased the above mentioned parameters and reduced testes weight compared to NDL groups. During RAP, LD reduced all the immunological parameters when compared with NDL and SD experiencing groups of RIP and RAP phases. The LD group reduced the immunological parameters compared to RAP, suggesting that LD had always an inhibitory effect on immune status being independent of reproductive phases. The intensity of the stimulatory effects of SD and inhibitory effects of LD during both reproductive phases was significantly different. We exposed another set of squirrels to the above photoperiodic schedule for prolonged period (30 weeks) during RAP. A clear testicular refractoriness followed by immunorefractoriness was observed in the group experiencing SD and LD for 30 weeks. The photorefractoriness presented by the testes was inversely related to the immunorefractoriness. The peripheral melatonin level of those squirrels reflected the photoperiodic signal perceived by squirrels for immunomodulation and gonadal function, suggesting that immune system and gonadal function might have coevolved.

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Seasonal changes in the immune system are evident in many vertebrate species and have been hypothesised to evolve to facilitate the survival in terms of annual cycles in pathogen prevalence and energy availability (1, 2). 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 day length induce not only changes in reproduction, but also changes in immunity (3, 4).

Melatonin, a hormone from the pineal gland, is a biological signal of day length and is a well-established modulator of immunity (5, 6). It has been proposed that melatonin plays a pivotal role in seasonal adjustments of immunity in tropical rodents (7). Earlier, it was 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 (3, 7), although, in tropical zones, the differences in summer and winter day length are much less (1 h and 45 min). It is now well established that a short day length enhances melatonin secretion and melatonin in turn induces immunity in most vertebrates, including humans (8).

Virtually nothing is known about the effects of ambient and experimental photoperiod on seasonal immune function of any seasonally breeding rodent. The present study aimed to examine the influence of different photoperiods on cell-mediated immunity and on other immunological parameters [lymphocytes, leukocytes, percentage stimulation ratio (%SR)] in a seasonally breeding tropical rodent male Indian palm squirrel, *Funambulus pennanti*, exposed during two crucial phases of reproduction (i.e. reproductively active and inactive) to investigate the following objectives: (i) is the immune status of the tropical seasonal breeding rodent *F. pennanti* photosensitive; (ii) does the effect of different photoperiodic schedules on immune activity differ during two crucial reproductive phases when there is very little difference in daylength of both reproductive phases; (iii) what is the interaction of melatonin and testosterone in terms of reproduction and immune regulation; and (iv) does the immune system, similar to gonads, show any spontaneous regression or refractoriness to prolonged photoperiodic exposures?

Material and methods

Animals

Indian Palm squirrel *F. pennanti* is a seasonal (long day) breeder of Indian origin. They are semi domestic in nature. Details of its behaviour and habitat, along with the reproductive pattern, has been reported previously (9). Adult male squirrels (average weight 100 ± 10 g) were collected from the vicinity of Varanasi (25°,18′N; 83°,1′E) and acclimatised to laboratory conditions for 1 week in a room fully exposed to ambient conditions and were kept in wire net cages (n = 7.) (635 × 635 × 762 mm in size). Because the experiment continued for a long period (10 and 30 weeks), natural protective measures such as dry grasses and hay sticks were provided during the winter. Soaked gram seeds (*Cicer arietinum*) were provided as food along with seasonal nuts, fruits, etc., and water was available *ad libitum.* All the experiments on the animals were conducted in accordance with Institutional practice and within the framework of revised Animal (Specific Procedure) Act of 2002 of the Goverment of India on animal welfare.

Photoperiodic treatment/exposure

Randomly selected seven adult male squirrels were exposed to a light condition equivalent to natural day length (NDL; 12 : 12 h light/dark cycle) and treated as the control, Group II had seven male squirrel exposed to a short day length (SD; 10 : 14 h light/dark cycle) and group III containing seven male squirrels exposed to a long day length (LD; 16 : 8 h light/dark cycle). Squirrels were maintained in the above conditions for 10 weeks. This experiment was repeated during the reproductively active (June; approxinately 14 h of light in natural conditions; RAP) and inactive (November; approxinately 10 h of light in natural conditions; RIP) phases. To study the prolonged effect of photoperiodic exposure, another set of experiments with 42 squirrels in each of photoperiodic group (i.e. NDL, SD and LD) was maintained up to 30 weeks (i.e. March to October RAP). Seven animals were killed after 0, 6, 12, 18, 24 and 30 weeks of exposure. Squirrels were weighed and killed at 22.00 h by decapitation at the end of each experiment. Trunk blood was collected in a heparinised tube. Serum was separated and stored at −20 °C.

Somatic and reproductive measures

Body weights of all the animals were noted initially and at the end of the experiment. Relative spleen, thymus and testes weight was recorded. Spleen and thymus was immediately processed for measurement of blastogenic response.

Assessment of total leukocyte and lymphocyte count

Total leukocyte (TLC) and lymphocyte (%LC) (n/mm^3) counts from peripheral blood were measured after Leishman staining as described previously (10).

Blastogenic response

Blastogenic response in terms of the % SR of lymphoid organs (spleen and thymus) was noted against the tritiated thymidine [^{3}H] count of untreated viable cells versus those treated with concanavalin A (Con A) (11). Cultures were harvested at the end of 72 h. The radioactivity was counted as counts per minute in a β-liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA) as described by Pauly and Soakal (12), as well as reported in detail elsewhere (7).

Delayed type hypersensitivity (DTH)

At week 10 of the first set of experiments, DTH was induced by application of the antigen oxazolone (Sigma, St Louis, MO, USA) to the ear pinna of squirrel following initial immunisation to the abdominal region (5% wt/vol). Oxazolone [0.5% (wt/vol) in 4 : 1, acetone: olive oil] was applied to the dorsal surface of right ear. The left ear was treated with vehicle alone. Ear swellings were measured every 24 h for the next 3 days. All measurements were made on relatively same region of the ear pinna of the experimental animals. A change in percent thickness was measured by comparing differences between treated and nontreated ear pinna (13–15).

Hormonal analysis

Radioimmunoassay (RIA) of melatonin was performed as described previously (16, 17) using Guildhey antisera (Guildhey, Guildford, UK). The validation of the RIA was also performed as described previously (17, 18). The intra- and inter-assay variation for melatonin was 9% and 15%, respectively. The sensitivity for melatonin RIA was 18–20 pg/ml. Testosterone RIA was performed by using Commercial kit (Lecco Dia, Inc., MO, USA). The intra- and inter-assay variation for testosterone was 4.5% and 5.6%, respectively, and the sensitivity was 6 pg/ml. The recovery of melatonin and testosterone RIA was 92% and 95%, respectively.

Statistical analysis

Statistical analysis of the data was performed with one-way ANOVA followed by the Student–Newman–Keuls multiple range test. $P < 0.05$ was considered statistically significant.

Results

Effect of different photoperiodic exposures

Effect on body, testes, spleen and thymus weight during RAP and RIP

There was no significant difference at all in body weights of the NDL squirrels from the initial and final data. Therefore, the final body weights of the NDL squirrels were taken into account and presented for comparison with the final body weights of other groups. Group I (NDL) had no difference in body weight during both the reproductive phases. SD reduced body weight during RAP, whereas LD increased the body weight during both reproductive phases (Fig.1A).

The relative spleen mass significantly increased in the squirrels under SD during both the reproductive phases, whereas LD reduced spleen weight during both phases compared to squirrels experiencing NDL (Fig. 2A). Similarly, relative thymus weight was parallel to that of the spleen weight during both reproductive phases (Fig. 2B).

Relative weight of the testes of the squirrels reduced during RIP. SD significantly reduced the testes weight during both reproductive phases; similarly, LD increased testes weight during both reproductive phases (Fig. 1B) compared to the NDL control.

Effect of photoperiod on immune status

Under SD, %LC during both phases of reproduction increased, whereas LD reduced it significantly only during RAP. Such a significant reduction was not observed during RIP (Fig. 3A). TLC presented parallel data to that of %LC for both reproductive phases (Fig. 3B).

When the splenocytes were challenged with Con A during RAP and RIP, an increase in % SR of splenocytes was noted under SD during both reproductive phases and a reduction in % SR of splenocytes was noted in squirrels experiencing LD (Fig. 4A) compared to NDL. There was an increase in % SR of thymocytes under

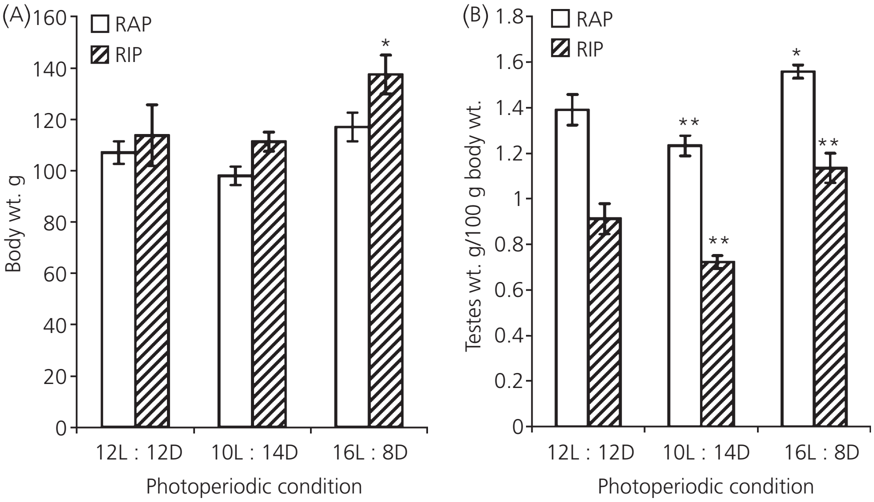

Fig. 1. (A, B) Effect of different photoperiods (12 : 12 h, 16 : 8 h and 10 : 14 h light/dark) on body and relative testes weight of Indian palm squirrel *Funambulus pennanti* during reproductively inactive (RIP) and active (RAP) phases (n = 7, mean ± SEM). The RIP and RAP groups of 12 : 12 h light/dark were compared separately with RIP and RAP groups of other experimental photoperiods. Vertical bars on each histogram represent the SE. Significant difference: *P < 0.05 and **P < 0.01.

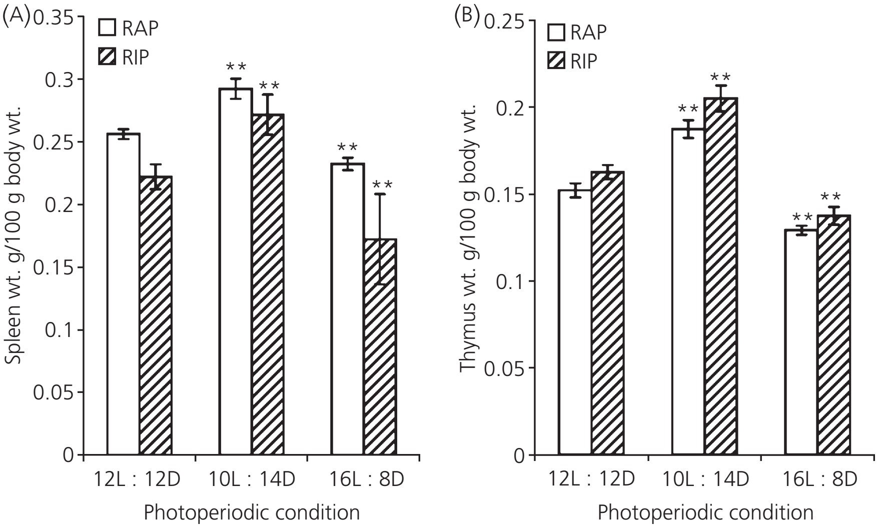

Fig. 2. (A, B) Effect of different photoperiods (12 : 12 h, 16 : 8 h and 10 : 14 h light/dark) on relative spleen and thymus weight of Indian palm squirrel *Funambulus pennanti* during reproductively inactive (RIP) and active (RAP) phases (n = 7, mean ± SEM). The RIP and RAP groups of 12 : 12 h light/dark were compared separately with RIP and RAP groups of other experimental photoperiods. Vertical bars on each histogram represent the SE. Significant difference: **P < 0.01.

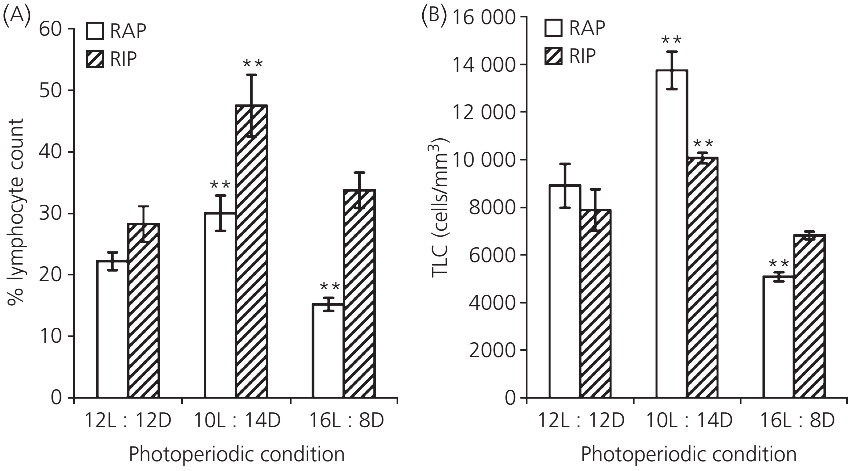

Fig. 3. (A, B) Effect of different photoperiods (12 : 12 h, 16 : 8 h and 10 : 14 h light/dark) on percentage lymphocyte and total leukocyte count of Indian palm squirrel *Funambulus pennanti* during reproductively inactive (RIP) and active (RAP) phases (n = 7, mean ± SEM). The RIP and RAP groups of 12 : 12 h light/dark were compared separately with RIP and RAP groups of other experimental photoperiods. Vertical bars on each histogram represent the SE. Significant difference: **P < 0.01.

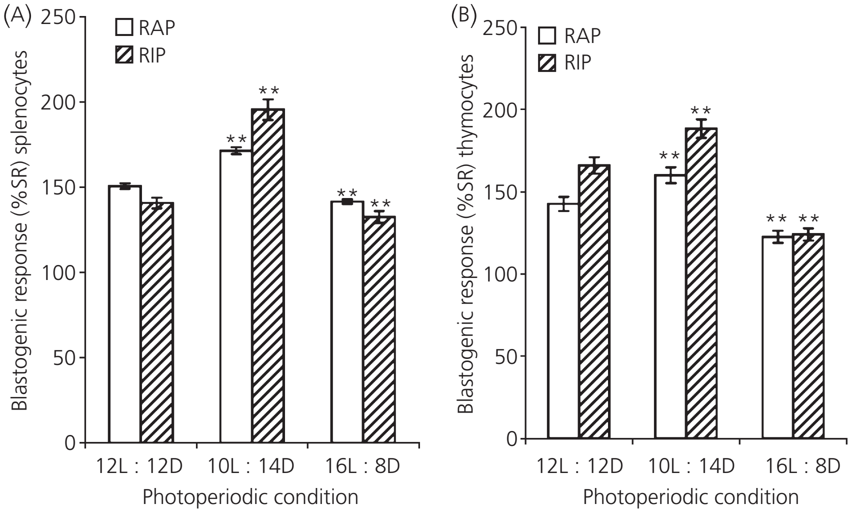

Fig. 4. (A, B) Effect of different photoperiods (12 : 12 h, 16 : 8 h and 10 : 14 h light/dark) on blastogenic response in terms of percentage stimulation ratio (%SR) of splenocytes and thymocytes of Indian palm squirrel *Funambulus pennanti* during reproductively inactive (RIP) and active (RAP) phases (n = 7, ± SEM). The RIP and RAP groups of 12 : 12 h light/dark were compared separately with RIP and RAP groups of other experimental photoperiods. Vertical bars on each histogram represent the SE. Significant difference: **P < 0.01.

SD, whereas LD reduced the same during both reproductive phases compared to NDL (Fig. 4B).

DTH was recorded significantly high in SD squirrels during both RAP and RIP compared to LD and NDL squirrels (Fig. 5).

Hormonal analysis

We observed significantly high peripheral testosterone level during RAP in NDL and LD squirrels compared to SD groups. On the other hand, testosterone was high in the LD group but the values were significantly lower during RIP (Fig.6A). By contrast, the melatonin level (night time; approximately 22.00 h) was significantly higher in the SD group of both reproductive phases compared to NDL and LD squirrels. The basal night time level of melatonin was high during RIP compared to RAP (Fig. 6B).

Effect of prolonged exposure (30 weeks) of photoperiodic schedule

Effect on spleen and thymus weight

Periodical sacrifice of animals at every sixth week presented an increase in spleen and thymus weight parallel up to 12th week under SD until week 24. However, after week 24, both lymphoid organs

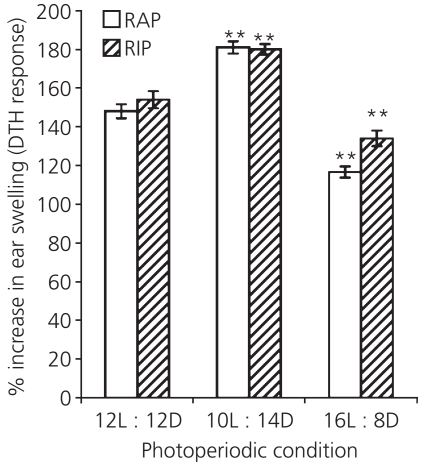

Fig. 5. Effect of different photoperiods (12 : 12 h, 16 : 8 h and 10 : 14 h light/dark) on delayed type hypersensitivity (DTH) response showing percentage increase in ear swelling of Indian palm squirrel *Funambulus pennanti* during reproductively inactive (RIP) and active (RAP) phases (n = 7, mean ± SEM). The RIP and RAP groups of 12 : 12 h light/dark were compared separately with RIP and RAP groups of other experimental photoperiods. Vertical bars on each histogram represent the SE. Significant difference: $^{**}P < 0.01$.

decreased their weight significantly compared to the NDL group. LD started decreasing the spleen and thymus weight from week 12 until week 24, and then increased the weight of both lymphoid organs even under LD conditions. A nonsignificant change in spleen and thymus weight was observed up to week 24 in NDL squirrels, whereas it also showed a nonsignificant decrease at week 30 (Fig. 7A, B).

Effect on testes weight

Testes weight increased significantly in LD squirrels compared to other photoperiodic groups for up to 24 weeks, whereas SD decreased testes weight up to 24 weeks compared to NDL and LD groups. At week 30, a significant increase in testis weight of 10L : 14D experiencing squirrels were observed (Fig. 8).

Effect on blastogenic response

Blastogenic response of SD squirrel splenocytes and thymocytes showed an increase up to week 24 and then declined significantly at week 30. The splenocytes and thymocytes of NDL squirrels presented no significant change in %SR up to week 30, whereas there was a decrease in the %SR of splenocytes and thymocytes of groups under LD, and a significant increase at week 30 (Fig. 9A, B).

Discussion

Using a seasonal breeding rodent, *F. pennanti,* of Indian tropical origin as a model, we report the photoperiodic regulation of immunity during two crucial reproductive phases (RAP and RIP) occurring at two extremely different environmental situations (i.e. summer and winter). During these phases, the basal level of two major hormones, melatonin and testosterone, were different and inversely related (9). Most of the time throughout the year, a squirrel experiences approximately 12 h of light; hence, we selected this photoperiod as the control. Furthermore, the winter photoperiod noted so far in the region of Varanasi is approximately 10 h of light, so the short photoperiod selected comprised 10 h. The longest photoperiod experienced by squirrel at Varanasi is approximately 14 h of light. To be on safer side when working with a long day breeder, we exposed them to a longer photoperiod of 16 h. Furthermore, we conducted a long duration experiment beginning from June to January for RAP, when the photoperiod in nature becomes long (approximately 14 h of light) to short (approximately 10 h of light; November/December) and then long (13 h of light; January). Similarly, in RIP (October to February), a change in photoperiod occurs

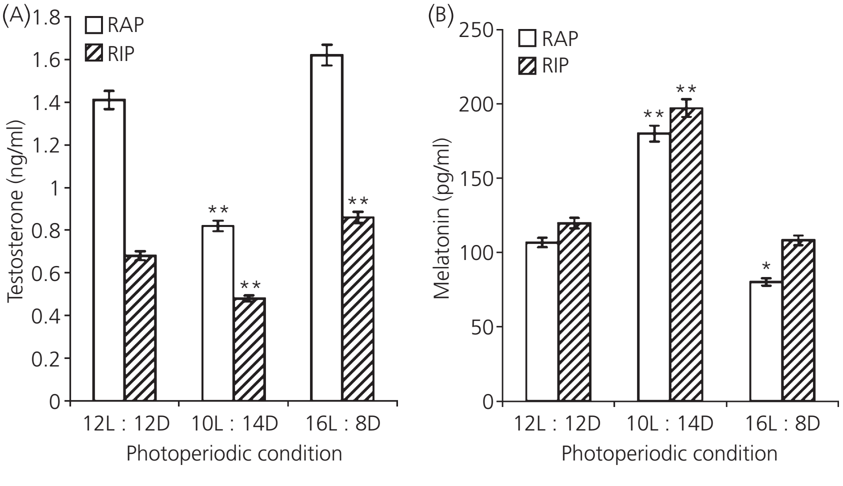

Fig. 6. (A, B) Effect of different photoperiods (12 : 12 h, 16 : 8 h and 10 : 14 h light/dark) on peripheral testosterone (ng/ml) and melatonin (pg/ml) level of Indian palm squirrel *Funambulus pennanti* during reproductively inactive (RIP) and active (RAP) phases (n = 7, mean ± SEM). The RIP and RAP groups of 12 : 12 h light/dark were compared separately with RIP and RAP groups of other experimental photoperiods. Vertical bars on each histogram represent the SE. Significant difference: $^{*}P < 0.05$ and $^{**}P < 0.01$.

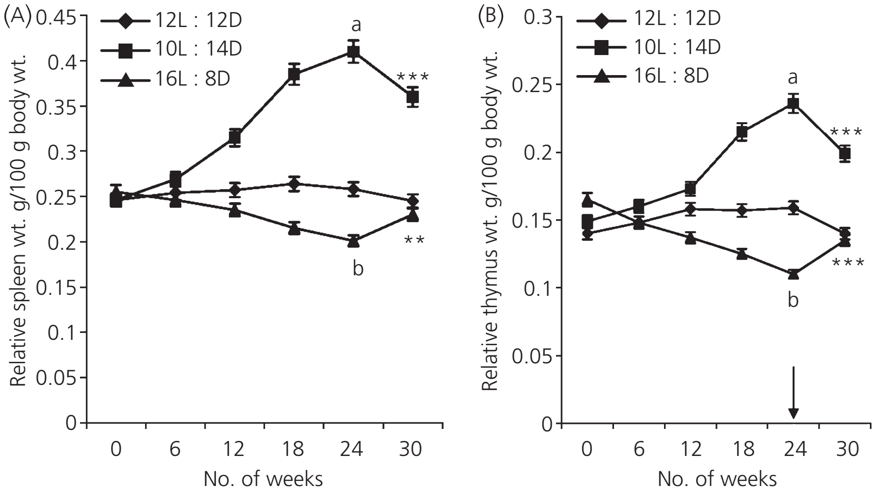

Fig. 7. (A, B) Prolonged exposure of photoperiod on relative spleen and thymus weight of *Funambulus pennanti* (n = 7, mean ± SEM). Vertical bars on each point represent the SE. ***P ≤ 0.001 and **P ≤ 0.01 indicate a significant difference between weeks 24 and 30. (a) P ≤ 0.001 indicates a significant difference between 12 : 12 h light/dark and 10 : 14 h light/dark and (b) P ≤ 0.001 a indicates significant difference between 12 : 12 h light/dark and 16 : 8 h light/dark during week 24 (↓, beginning of refractoriness).

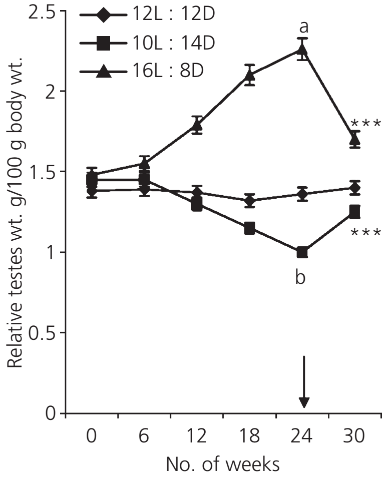

Fig. 8. Prolonged exposure of photoperiod on relative testes weight of *Funambulus pennanti* (n = 7, mean ± SEM). Vertical bars on each point represent the SE. ***P ≤ 0.001 indicates a significant difference between weeks 24 and 30. (a) P ≤ 0.001 indicates a significant difference between 12 : 12 h light/dark and 16 : 8 h light/dark and (b) P ≤ 0.001 indicates a significant difference between 12 : 12 h light/dark and 10 : 14 h light/dark during week 24 (↓, beginning of refractoriness).

from short (approximately 10 h of light) to long (approximately 13 h of light) in nature. To overcome the problem of the photoperiodic transitional changes in nature, we selected 12 h of light/dark as the control photoperiod, although all other ecofactors (i.e. temperature and humidity) remained the same as in the ambient condition.

Photoperiodic regulation of neuroendocrine activity (i.e. control of melatonin synthesis) is another important physiological function known to regulate both reproduction and immunity in various species of mammals (2, 19, 20). In other words, melatonin is taking care of immunity when the other peripheral metabolic hormonal (testosterone and thyroxin) levels are low, a situation generally observed during RIP in seasonal breeders (21).

In our experiment SD exposure during RAP to squirrels was quite similar to the winter months and, hence, a reduced gonadal activity and increased immune status was noted. Here, it could be suggested that the winter adaptation of immunity of the squirrel is under neuroendocrine control. We observed an increase in TLC and %LC of squirrels under SD exposure, whereas a decrease was noted in LD squirrels. This is in line with the hypothesis that SD might correspond to the winter season, with less gonadal activity, low testosterone and high melatonin and immunity levels (19). This high melatonin level is considered to be an immune enhancer helping the squirrels survive under winter stress (i.e. less food and shelter) (3, 19) demonstrating that SD was effective in mediating immune function in individuals with less reproductive response (22). The present study also suggests that immune function is associated with photoperiodic control of the neuroendocrine mechanism, which involves the hypothalamus pituitary and pineal gland. Hence, an interaction between light influencing the pineal gland and the secretion of melatonin might be acting on the hypothalamus and pituitary to release follicle-stimulating hormone/luteinising hormone, leading to high/low testosterone secretion by the testes (23, 24). This could be responsible for melatonin because receptor subtypes (MT1 and MT2) for melatonin hormone were recorded on the pars tuberalis and different areas of the hypothalamus (25). It is known that the melatonin rhythm transduces information about day length to regulate hypothalamic gonadotrophin secretion (26, 27). In the present study, the SD induced rise in melatonin might be the signal that suppressed the neuroendocrine secretion of gonadotrophin-releasing hormone and led to gonadal regression of squirrels under SD compared to the enlarged testes of the squirrels under LD.

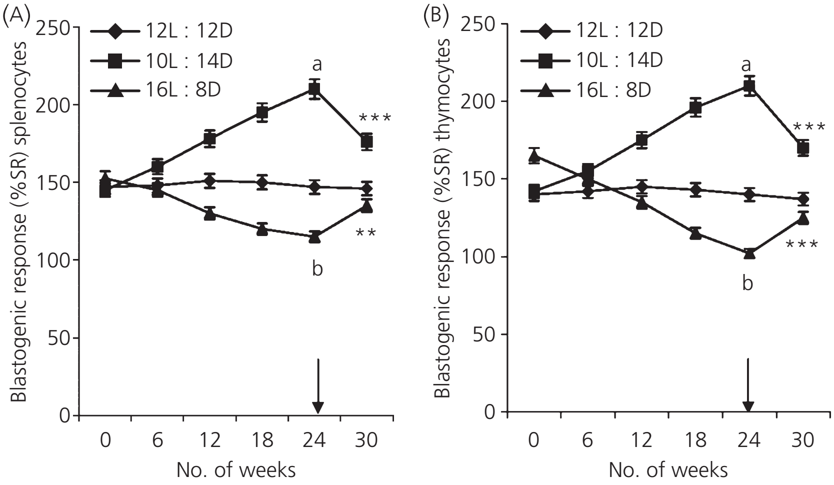

Fig. 9. (A, B) Prolonged exposure of photoperiod on percentage stimulation ratio of splenocytes and thymocytes of *Funambulus pennanti* (n = 7, mean - SEM). Vertical bars on each point represent the SE. ***P ≤ 0.001 and **P ≤ 0.01 indicate a significant difference between weeks 24 and 30. (a) P ≤ 0.001 indicates a significant difference between 12 : 12 h light/dark and 10 : 14 h light/dark and (b) P ≤ 0.001 indicates a significant difference between 12 : 12 h light/dark and 16 : 8 h light/dark during week 24 (↓, beginning of refractoriness).

The photoperiodic effects on immune cell function might comprise a similar neuroendocrine mechanism driving the photoperiodic control of reproduction, on the one hand (28), and immunity, on the other, with the help of two inversely related hormones in the system (i.e. melatonin and testosterone). SD induced high melatonin, and enhanced both humoral and cell mediated immunity in squirrels, as noted previously in some species of rodents (29, 30). Melatonin has been found to activate T helper cells, which accounts for the rise in the stimulation of the blastogenic response observed in our study. A significant increase in %SR of splenocytes of SD animals was recorded with respect to LD and NDL squirrels. Lymphocytes were large in number in SD squirrels compared to LD squirrels. Because, resources are more limited in tropical winter than at other times of the year, augmentation in basal activity by some lymphocyte populations in our squirrels under SD may ensure an accelerated response to any infection through the activation of memory cells.

DTH responses require the formation of memory T cells after an initial exposure to oxazolone. Photoperiod affects both the formation (encoding) and the functional expression (retrieval) of this immunological memory in our squirrel model. Our data show a marked increase in the DTH response under SD conditions, which accounts for the formation of T helper memory cells in the body, which, in turn, is responsible for antibody production and heightened immune response against any sort of foreign antigen. It is not clear whether seasonal photoperiodic timing regulates the immune function or whether it enhances immune function or sustains indefinitely under SD and or winter conditions. To check this possibility, we exposed the squirrels to SD and LD for a prolonged period.

An interesting result was obtained after prolonged exposure to photoperiod for 30 weeks. Up to 24 weeks, SD stimulated the weight of the lymphoid organs and %SR, whereas LD reduced the same. At week 30, there was an inverse response of immune status to their respective photoperiods (i.e. spontaneous reduction in immune status was noted in squirrels under SD, whereas a spontaneous increase in immune status was noted in squirrels experiencing LD). Hence, we suggest that enhanced immune function (experimental or in nature) is not indefinite and, therefore, almost all immune parameters showed spontaneous regression comparable to refractoriness. This response was quite similar to the response of testes weight, which also presented a photorefractoriness. We suggest that prolonged exposure to photoperiodic conditions not only leads to reproductive refractoriness, but also lead to immune refractoriness. Furthermore, Freeman and Zucker (31) reported that refractoriness to melatonin occurs independently at multiple brain sites in hamsters. Such refractoriness to melatonin can be compared with our results of photoperiodic induced refractoriness as a different photoperiod is responsible for the induction of different levels of internal melatonin.

In summary, the present data suggest an important physiological and adaptive significance of photoperiod-mediated immune function in a tropical rodent. The photoperiodic time-keeping mechanism regulating seasonal transition in reproduction and immune function helps the organism to survive and perpetuate (32). The net effect of elevated immunity under SD appears to counteract the suppressive effects of environmental stresses, such as low ambient temperatures or reduced food availability in winter. Furthermore, the interaction between environmental factors and immunity must be considered when explaining seasonal adaptations in wild rodent animals. Tropical rodents, although living in an environment with a small variation in photoperiod from summer to winter compared to the temperate zone rodent, also use this slight change in photoperiod as a signal to check seasonal changes and adjust their immune status accordingly. Furthermore, prolonged photoperiodic exposure may lead to spontaneous regression/stimulation of immune status, which may be termed immune-refractoriness. Our finding that short day length enhances immune function complements melatonin as an immune enhancer, whereas gonadal hormones act as an immune suppressor and are responsible for a trade-off relationship between these two important hormones. Photoperiod, which induced reproductive refractoriness in the squirrel, was sufficient to induce immunorefractoriness. This clearly supports our suggestion the reproduction and immunity has coevolved both morphologically and functionally.

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