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Correlation between Annual Immunity, Reproductive Success and Melatonin in a Nocturnal Owlet, *Athene Brama*

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Abstract: *Seasonal fluctuations in immune system and reproduction are considered of great adaptive importance in mammals but such studies are lacking in avian, especially for any nocturnal bird. We accessed the seasonal variation in immune status of a tropical nocturnal owlet, Athene brama in relation with environmental factors and reproduction. For immune status we measured the total leukocyte count (TLC), percent lymphocyte count (%LC) and blastogenic response of peripheral blood mononucleated cell (PBMC) from blood. Maximum immune status and low gonadal activity (as measured by laprotomy) coincided with high circulating level of melatonin during the July and August months with prevailing monsoon condition in nature. Correlation analysis showed a less positive relation between melatonin and %SR of PBMC in both male and female owlets. However, a strong negative correlation was observed between testosterone, estradiol and %SR of PBMC. General immune status and melatonin level both showed an inverse relationship with gonadal activity suggesting that in this owlet melatonin is acting as immunostimulator and balancing immunity and reproduction. Hence, it could be suggested that in Indian spotted owlet, A. brama both immune and reproductive system might have coevolved sharing a bidirectional relationship to maintain physiological homeostasis.*

Keywords: *Seasonal, Immune status, Melatonin, Reproduction, Owlet*

1. INTRODUCTION

The Indian spotted owlet, *Athene brama* is commonly known as "farmer's friend" because of its biological importance in predating on the field rodents, mice, lizards and other small pests of agricultural crops. The frequent use of herbicide and pesticide directly or indirectly influences immunity of these birds via the food chain [1]. The nature of the food always challenges the immunity and this could be the reason behind the decreasing number of many predator birds. Several studies on spotted owlet have inferred that habitat quality, prey abundance and weather are having drastic influence on reproduction [2],[3],[4]. However, only few studies were existing dealing with reproduction of birds in relation with immune capacity under natural condition [5],[6],[7],[8],[9],[10]. In addition owlets have to endure many stressors in their natural environments for e.g. food shortage, predator pressure, high parasite densities with season, and social pressure [11], [12], [13].

Evidence has accumulated that melatonin, a chemical messenger of photoperiod and phase response allows seasonal breeders to anticipate the changing seasons and make the necessary adjustments in advance of the actual breeding period [14]. It has been reported that melatonin, plays a pivotal role in seasonal adjustments of immunity in diurnal birds [9], [15]. Information is lacking for any nocturnal avian species where melatonin secretion is mostly progonodatropic in nature [11], [12], [13] but, has not correlated reproduction with immune function. In this regard the interplay of gonadal steroid – testosterone and melatonin could be of great interest because of their opposite functional nature towards immunity, testosterone acts as immune suppressor and gonado-stimulator while melatonin acts as immunostimulator and gonado-suppressor to maintain physiological and adaptive homeostasis in birds., We, therefore studied the correlation between peripheral gonadal steroid and melatonin in modulation of annual immune status of a nocturnal owlet, *Athene brama.*

2. METHODS

2.1. Animal Care and Its Maintenance

A. brama, commonly known as Indian spotted owlet belongs to the order – Strigiformes. They are abundant at the vicinity of Varanasi (Lat 25^0 18' N; Long 83^0 1' E). Adult spotted owlet *A. brama* (body length, snout to vent-19-21 cm; body weight~110-120 g) were collected during the last week of each month for annual study. The entire collected owlets were acclimatized in an outdoor aviary for two weeks before any experiment. They were fed with fresh small rodents, meat and water *ad libitum*. Wooden hutments and artificial burrows were provided for hiding during the daytime. After acclimatization to the laboratory conditions, five owlets of each sex were randomly collected numbered (ring with number on left leg) and blood was withdrawn from left pectoral wing vein. It was replaced with 1ml of saline injection on other right pectoral vein and after experimentation they were released in nature.

All experiments were conducted in accordance with institutional practice and within the framework of Animals (Scientific procedure) Act of 2002 of Government of India on Animal Welfare.

2.2. Sampling

Blood collected from pectoral vein of five selected and numbered owlet of each sex during night time (~21 h) under dim red light, part of which was processed for TLC,%LC and %SR of PBMC while remaining blood was centrifuged and plasma was stored at –20 ^{0} C until RIA for melatonin, estradiol and testosterone. Laprotomy was performed to measure the gonadal function as judged by measuring the gonadal volume by formula, $4\pi ab^2$ where a=long axis and b=short axis of testes, then changing it into gonadal weight by using the formula of Watson-Whitmyre and Stetson (1985) following pre-determined linear regression formula for specific animals during different phases of reproductive cycle.

2.3. Hematological Parameters

The blood cell count (TLC) and percent lymphocyte count (%LC) were done following non invasively drawn blood from pectoral vein. The TLC and % LC are clinically important parameter for first line defense of innate immunity [8]. Blood was taken in a WBC pipette and diluted 20 times with Turk's fluid (2.0 ml Glacial acetic acid, 0.1 g mercuric chloride, one drop Aniline, and 0.2 g Gention violet). The white blood cells counted (no./mm3) in Neubauer's counting chamber (Spencer, USA) under the Nikon binolocur microscope. Thin blood film was stained with Leishman's stain for differential lymphocyte count under oil immersion Leitz MPV3 microscope. Lymphocyte counts (no. /mm3) was determined from the total and differential lymphocyte count by using the following formula:

$$\text{Lymphocyte Count} = \frac{\text{TLC} \times \text{Lymphocyte percentage}}{100}$$

2.4. Blastogenic Response of Peripheral Blood Mononucleated Cell (PBMC) and Percent Stimulation Ratio (%SR)

2.4.1. Isolation of Cell for Proliferation Assay

A modified method as described elsewhere [16] was used for isolation of PBMC. PBMC were isolated from the heparinized blood collected from pectoral vein in a 1.5 mL tube. Blood was diluted in 1:1 ratio with PBS and under layered with Ficoll hicep^{TM} LSM (Himedia Lab, India). White ring of mononuclear cells collected, washed with PBS, centrifuged and suspended in RPMI 1640 supplemented with 10% fetal calf serum and 100 units of penicillin and streptomycin. The cell concentration was adjusted to $1x10^7$ viable cells/mL and plated in 96 well plate in duplicate having concentration of (2×10^6cells/well) for the measurement of cell proliferation by MTT assay and OD values at 570 nm were taken and the %SR was calculated.Two milliliters of cell suspension was placed in duplicate sterile culture plates (Nunc ^{TM}, Denmark, Cat. No. 153066, Diameter-3.5cm) and kept at 41°C in a 5% CO2 incubator for 72 h. The control culture plates were incubated without mitogen whereas test culture plates were incubated with mitogen concanavalin A (Con A; T cell mitogen; Sigma-Aldrich, USA; 10µg/mL). Eighteen hours before harvesting, 1µCi of tritiated thymidine (3H) (BARC, India; specific activity 8.9 Ci/mM) was added to each culture plate. Culture plates were harvested after 72 h of incubation following the method described elsewhere [17]; with

modification [18]. Blastogenic response was measured in terms of [3H] thymidine uptake against stimulation by Con A of the PBMC. For each bird, a duplicate culture was set.

$$\% \text{ SR} = \frac{\text{CPM with Con A}}{\text{CPM without Con A}} \times 100$$

2.5. Radioimmunoassay

Blood collected in heparinized tubes were centrifuged at 800 X g at 4 ^{o}C to obtain plasma. Plasma samples were then stored at -20 ^{o} C until assayed for estradiol, testosterone and melatonin by radioimmunoassay RIA. Estradiol and testosterone was estimated using the appropriate RIA kits from Immunochemical Corporation, Carson, USA. The melatonin RIA was performed as decribed elsewhere [19]. The validations for this bird were described earlier [12],[13]. For validation of melatonin RIA, we compared displacement curves obtained using synthetic standards with those of plasma samples from owlets, which showed clear parallelism. However, other variables such as assay variation, sensitivity and recovery in all the RIAs were also validated. The inter- and intra-assay variation for all the RIAs varied between 8 and 9.2% and 4.3 and 5% (n=5), respectively. The sensitivity for gonadal steroids RIA varied between 6 and 15 pg/ml, while the melatonin RIA sensitivity was approximately 18-20 pg/ml. The recovery of standards such as melatonin, estradiol and testosterone in different RIAs ranged between 85 to 92%.

2.6. Histology

During laprotomy a small portion of testes and ovary was sucked with help of cannula/lumber needle, this biopsy method does not causes any local injury to birds. The portion of testis and ovary was fixed in neutral formalin and processed for dehydration followed by paraffin infiltration. Sections of 5 micron (Leica Microsystem Inc., USA) thickness were spread on slides and stained with haematoxylin-eosin stain, observed under microscope (Leica MPV-3, Germany) and documented.

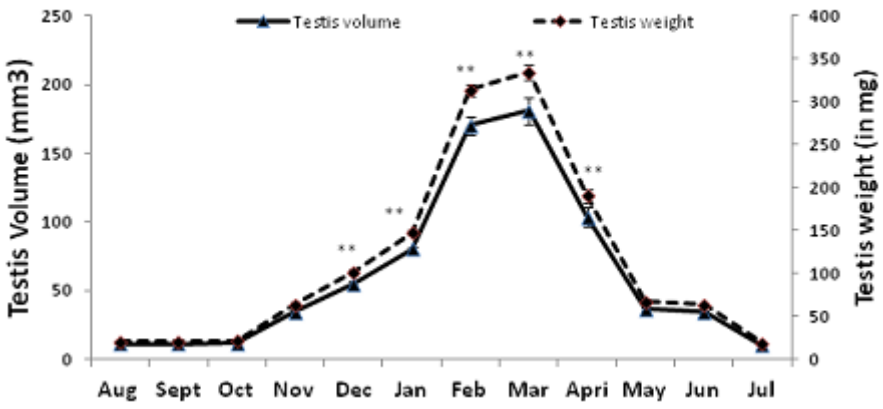
2.7. Statistical Analysis

The data was presented as means $\pm$ S.E. (M$\pm$S.E.) and were analyzed by two - way analysis of variance (Two Way repeated ANOVA) followed by post-hoc test - Dunnett t-test (2- sided). In Dunnett t- test, male was used as control and compared with female owlets. For annual variation data of June (summer) month was treated as control and compared with that of other months. The mean difference was considered significant at the $p \leq 0.05$ level. Correlation analysis was performed to determine the possible linear relationship between melatonin, testosterone and other immune parameters and expressed as Pearson coefficient (r). The data were depicted with the help of Microsoft Excel 2007 program.

3. RESULTS

3.1. Annual Variation in Gonadal Volume and Gonadal Weight

Fig. 1a



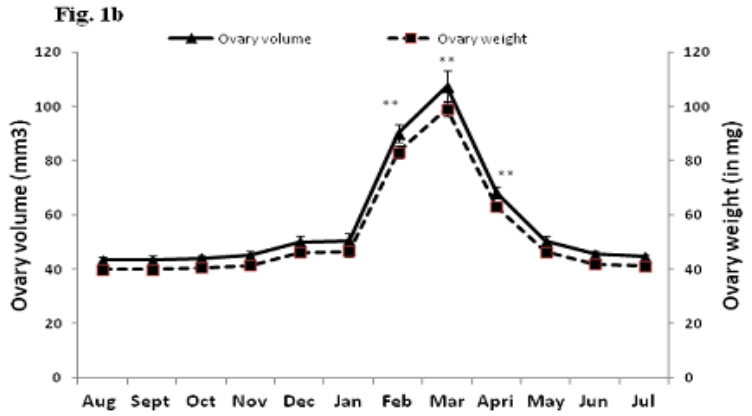


Fig1. *Annual variation of (1a) testicular volume (mm^3) and testicular weight (mg); (1b) ovarian volume (mm^3) and ovarian weight (mg) of Athene brama. Data of each point represent mean ± S.E.M; N=5. Vertical bar on each point represents standard error.***p <0.001. In Dunnett t-test, the data of June month were treated as control and compared with that of other months.*

A two way ANOVA results showed significant difference variation in gonadal volume as was found on sex (*df= 1, F= 72.197, p≤ 0.001*) as well as on months (*df= 11, F=189.627, p≤ 0.001*). However, no significant interaction was observed between sexes and months (*df= 11, F=11.618, p= 0.073).* High testicular volume along with testicular weight was observed during February (170 ± 6.9) – March (181± 9.7) being highest during March (181 ± 9.7) and the lowest testicular volume was observed in July (10 ± 0.9)-October (11.5 ± 1.1) corresponding to peak value of testicular weight as observed during March (334.11 ± 8.1) and minimum testicular weight during July (18.445±1.1)-October (21.214 ± 1.1) (Fig. 1a). Similarly ovarian volume was highest from February (90.2 ± 3.3) – March (107.2 ± 5.7) along with ovarian weight February (83.06 ± 2.3) – March (99.003 ± 2.7).The lowest ovarian volume was observed in June (45.4 ± 1.3) -November (44.2 + 1.9) corresponding to minimum ovarian weight June (41.94 ± 1.1) - November (41.5275 ± 1.7) (Fig.1b).

3.2. Annual Variation in Total Leukocyte Count (TLC) and %Lymphocyte Count (%LC)

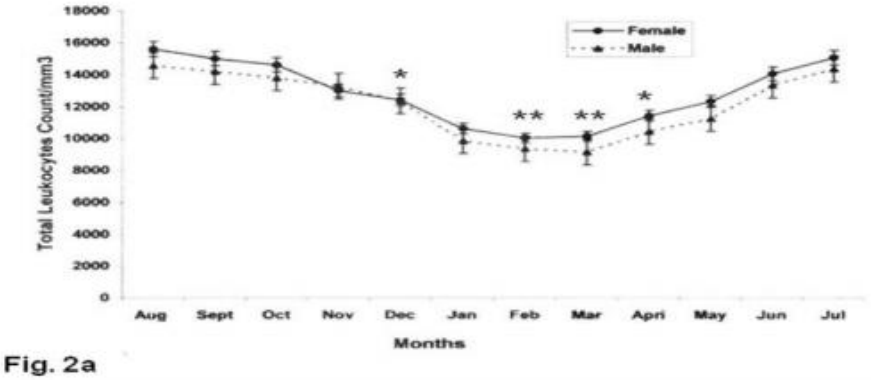


Fig. 2a

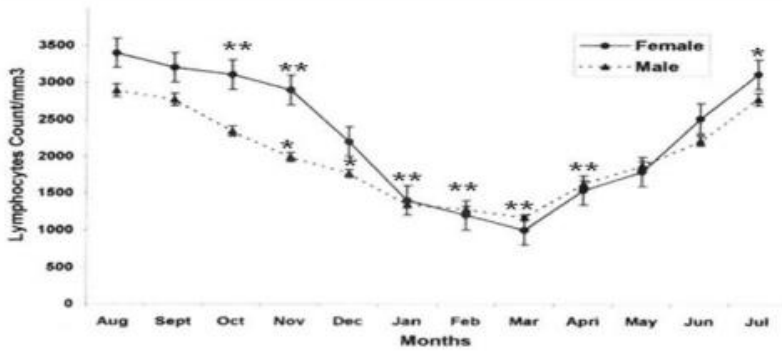


Fig. 2b

Fig2. *Annual variation in total leukocyte count (TLC) (2a) and %lymphocyte count (% LC) (2b) of male and female Athene brama .Data of each point represent mean ± S.E.M; N=5. Vertical bar on each point represents standard error. *p < 0.05 and **p <0.01. In Dunnett t-test, the data of June month were treated as control and compared with that of other months. Male v/s Female.*

An increased value of TLC during (June to October) was noted in both male and female owlet. The maximum value was observed in both sexes in August month, male (15030 ± 292.57 cells/mm^3) and female (14300 ± 396.23 cells/mm^3). A significant difference in TLC was found on sex (*df= 1, F= 33.135, p≤ 0.001*) as well as on months (*df= 11, F=67.901, p≤ 0.001*) but no interaction was observed between sexes and months (*df= 11, F=1.754, p= 0.073*). A significant decrease (p< 0.05) in the TLC was noted from January to March in both the sexes. A similar trend was observed for the % lymphocyte count (LC) being minimum in the month of March (male-1167± 21.2, female- 1008 ± 17.3 lymphocytes / 100 cells) and maximum was noted in the month of August for both male (2890± 29.23) and female (3400± 37.8). The variation in % LC among sexes (*df=1, F= 118.26, p≤ 0.001*), months (*df=11, F=275.138, p≤ 0.001)* and months versus sexes (*df=11, F=16.301, p≤ 0.001)* was significant (Fig.2a, b).

3.3. Annual Variation in Percent Stimulation Ratio (%SR) of PBMC and Circulatory Level of Melatonin

Blastogenic response was measured in terms of %SR of PBMC. A significant difference in %SR was noted for sex (*df=1, F= 60.63, p≤ 0.001)* as well as for months (*df= 11, F=43.29, p≤ 0.001*) and also interaction was observed between sexes and months (*df= 11, F=7.4, p≤ 0.001).* Lowest %SR was noted in male owlet in the month of January (163.4 ± 8.9) while in female it was noted in March month (160.1 ± 7.4). A gradual and parallel increase in %SR was noted in both sexes from April onwards till it reached to its peak value in August (244.6 ± 16.9), (240.1 ± 12.3) in both sexes respectively (Fig.3).

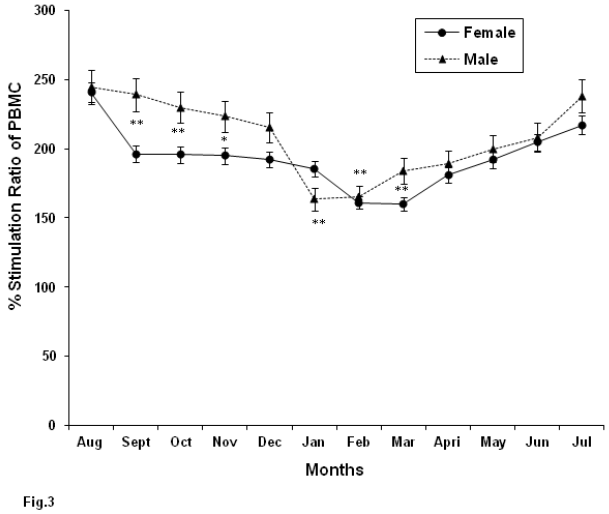


Fig.3

Fig3. *Annual variation in %stimulation ratio of PBMC of male and female Athene brama .Data of each point represent mean ± S.E.M; N=5. Vertical bar on each point represents standard error. *p < 0.05 and **p <0.01. In Dunnett t-test, the data of June month were treated as control and compared with that of other months. Male v/s Female.*

The variation in plasma melatonin was significant (*p≤ 0.001*) among (*df=1, F=162.588*) as well as months of the year (*df=11, F= 345.1*). The interaction between genders and months was also significant (*df=11, F=31.38, p≤ 0.001*). From June onwards there was a gradual rise in the value of plasma melatonin showing a small peak during August (220 ± 16.2; 145 ± 9.6) and a large peak in December (340 ± 18.7; 220 ± 11.1) in both the sexes respectively (Fig. 4a, 4b).

3.4. Annual Variation in Circulating Level of Sex Steroids (Testosterone and Estrogen)

Testosterone level in male was highest during the month of March (54 ± 6.3 ng/ml) and lowest during July (8.0 ± 2.7 ng/ml). The peak value of March was significantly high (p<0.05) when compared with other months. The peripheral estrogen level in female showed variation throughout the year, presenting highest value in the month of March (53.3 ± 2.7 pg/ml), while the lowest values was noted during May (8.9± 0.79) - August (7.3± 0.34) (Fig. 4a, 4b).

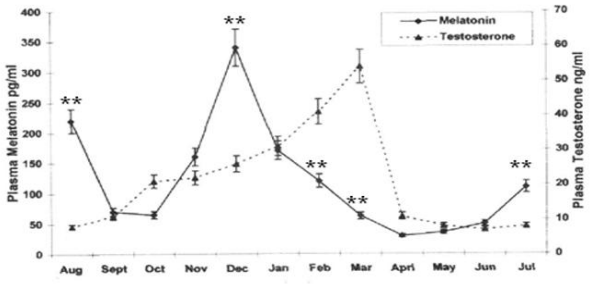


Fig. 4a

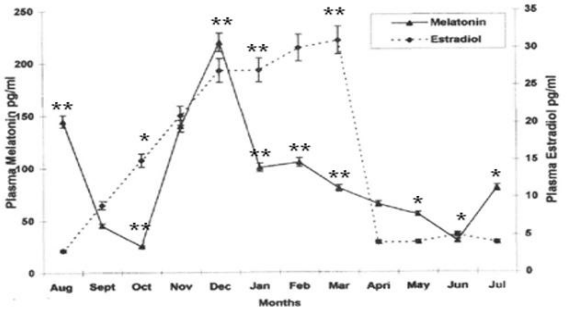


Fig. 4b

Fig4. *Annual variation in the level of (4a) plasma melatonin (pg/ml) and plasma testosterone (ng/ml) in male Athene brama (4b) plasma melatonin (pg/ml) and plasma estradiol (pg/ml) in female Athene brama. Data of each point represent mean ± S.E.M; N=5. Vertical bar on each point represents standard error.**p <0.01. In Dunnett t-test, the data of June month were treated as control and compared with that of other months. Male v/s Female.*

3.5. Histological Observation of Testis and Ovary During Reproductively Inactive and Reproductively Active Phase

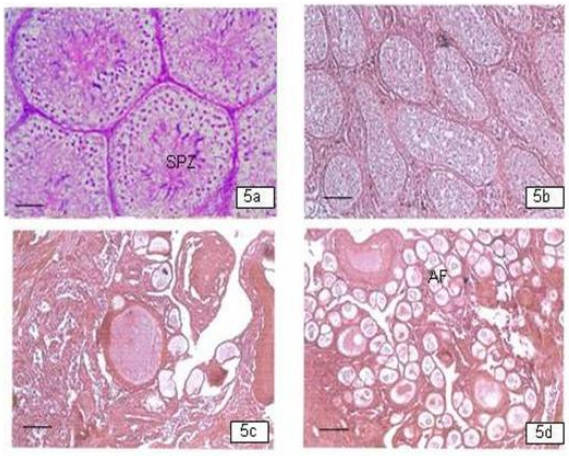


Fig.5

Fig5. *Histoarchitecture of testis during reproductively active and inactive phase of Athene brama. (5a and 5b). Histoarchitecture of ovary during reproductively active and inactive phase of Athene brama.(5c and 5d). (SPZ,spermatozoa and AF, atretic follicle). Scale bar = 50 µm.*

During reproductively inactive phase (June-October) a total arrest of spermatogenesis was noted in testis. More immature follicles along with atretic follicles were observed in the ovary during reproductively inactive phase (June-October). During reproductively active phase lumen full of spermatozoa was observed along with various advancing stages of spermatogenic germ cells in testis. Large number of mature and developing follicle were observed in ovary during reproductively active phase (Fig. 5a, 5b, 5c and 5d).

3.6. Correlation Analysis between Percent Stimulation Ratio (%SR) of PBMC, Melatonin, Testosterone and Estrogen Level

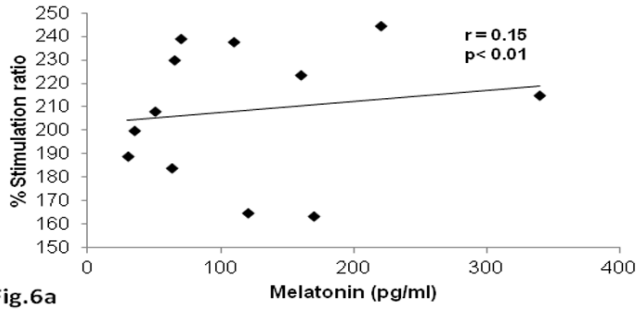


Fig.6a

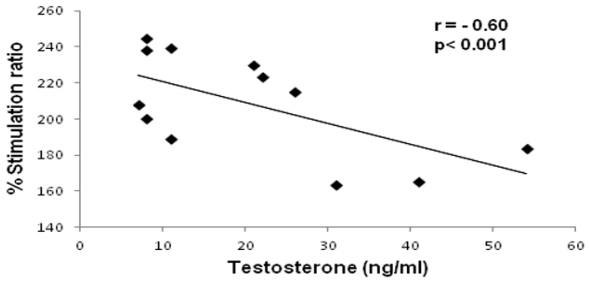


Fig.6b

Fig6. *Correlation analysis between (6a) melatonin and percent stimulation ratio (%SR) (r =0.15, p<0.01) (6b) testosterone and percent stimulation ratio (%SR) of PBMCs in male Athene brama (r = - 0.60, p < 0.001).*

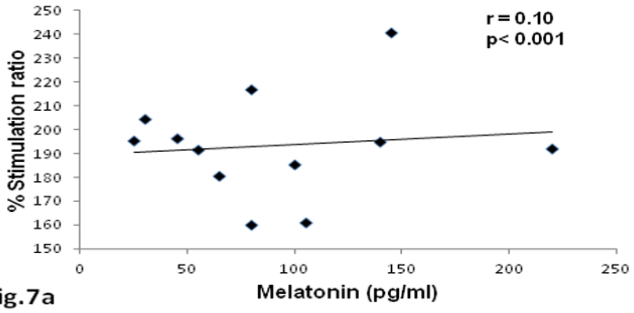


Fig.7a

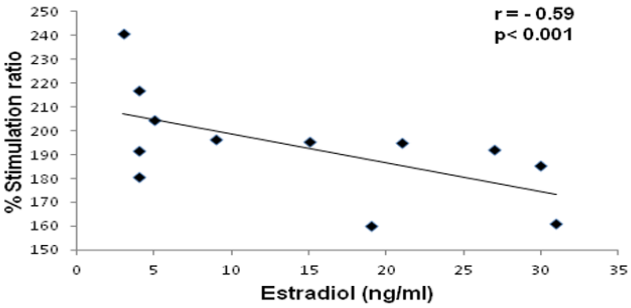


Fig.7b

Fig7. *Correlation analysis between (7a) melatonin and percent stimulation ratio (%SR) (r = 0.10, p < 0.001) (7b) estradiol and percent stimulation ratio (%SR) of PBMC in female Athene brama (r = - 0.59, p < 0.001).*

In male and female owlets, a positive correlation (r = 0.15, $p > 0.001$; r = 0.10, $p \leq 0.001$) between %SR of PBMC with melatonin level in annual cycle was noted (Fig.6a &7a). However, in male owlet a negative correlation (r = - 0.60, $p \leq 0.001$) was observed between %SR of PBMC and testosterone level (Fig.6b). In female owlets also a negative correlation (r = - 0.59, $p \leq 0.001$) was observed between %SR of PBMC and estradiol level (Fig.7b).

4. DISCUSSION

The result of the present study led us to propose that immunity and reproduction both are important physiological events as far as the perpetuation of any species is concerned. *Athene brama*, a nocturnal spotted owlet is distributed in Southern Asia and found abundantly in most of the Indian subcontinent. Commonly known as farmer's friend, it mainly preys upon beetles, moths, insects, lizards and small rodents which are crop pests. A detailed account of environmental and hormonal regulation of reproduction in spotted owlet has already been published earlier [13], [20] but the immune status of this bird was never explored. In the present study, we correlated the immunity and reproduction of owlets during both reproductively active and inactive phases being maintained under natural environmental conditions in our external aviary.

We found an inverse relationship between immune status as judged by the general immune parameters i.e. TLC, LC of blood and % SR of PBMC with that of reproductive function as judged by the gonadal volume and weight and peripheral testosterone, estradiol level in both the sexes of spotted owlets during reproductively active and inactive phases. In spotted owlets, the immune status was found lowest in both sexes in the month of Febuary - March when a ciculatory gonadal steroid was high and melatonin hormone level was moderate. This was because spotted owlet is a winter (short day) breeder (December-March) where melatonin might be acting as a progonadotropic hormone [12]. Immune status during this phase was moderately low because of high gonadal steroid level. High gonadal steroid though suppresses immune status but the birds remained healthy due to the anabolic support of the steroids. Our suggestion, gets support from the earlier reports of [21],[22],[23] suggesting that the costs of immune activity underlie the seasonal reproductive fluctuations [2],[3],[24],[25],[26].. However, the immune activity of spotted owlet was not presenting a complete 'trade-off' relation with reproduction as melatonin appeared to be progonadotropic.

Among all the ecological factors of tropical zone, we found that changes in season condition have impact on the modulation of immune status of birds and mammals [10],[27].In this bird, reproductively active phase occurs at the end of winter (Dec-March) and breeding is over before the onset of summer (i.e. before the long day length prevails). Owlets being nocturnal in habit do not like the long day length and hence, they enter into reproductively inactive phase summer (June-October). We observed a very high melatonin level in male than in female (peak value male ~ 340 pg/ml; peak value female ~ 220 pg/ml) during inactive phase as because in winter the level of melatonin is moderately high. This high peripheral melatonin is an important seasonal adaptation e.g., inhibition of reproductive functions and behavior along with initiation of immune functions [28] for fighting disease, or coping with other ecological stressors [29]. [30].

Indeed, the immune system can be modulated by neuroendocrine-gonadal axis [31]. In owlets, low gonadal activity was noted from April – August (summer and monsoon) coinciding with the moderate level of melatonin along with moderately high immune status. This high immune status was due to the less suppressive effect of gonad hormones on immune function of spotted owlets. The immunity needs to be high in summer and monsoon months to protect this predating bird from their food which are small rodents that may be infected during monsoon due to high humidity and temperature that promotes contamination and various infections in flesh of the dead animals upon which generally owlets feed. A low gonadal steroid and high melatonin level during the monsoon months (July – September) leads to high immune status being sufficient enough for this bird to protect them from infection which gets naturally promoted while feeding on small infected animal. Our results thus provide evidences that the immune system is linked not only to reproductive process but also to ecological stress, where melatonin plays an important role in conveying the environmental information to the reproductive-immune axis. Our suggestions get support from the studies on domestic fowl that showed strong antibody responses with low testosterone titres [32].

Substantial reports demonstrated that changes in day length induce not only changes in reproduction, but also changes in immunity [29], [30], [33], [34], [35]. Interestingly, we observed a significant

difference in immune status of male and female owlets during the winter months (January to November), while the rest of the year no such differences existed suggesting that high threshold level of gonadal steroid might be responsible for a change in immunity. Changes in immune activity are driven by short- and long-term fluctuations in peripheral hormone in seasonal breeders as the receptor distributions vary on the target tissue [36].

5. CONCLUSION

In conclusion, our study supports the fact that the immune, endocrine and nervous systems are intricately connected to serve to communicate information about the internal and external environments to control the body homeostasis in these owlets. This interrelation of melatonin and gonadal steroid in a nocturnal bird is an important seasonal adaptation to support immune function for survival during extreme harsh environmental conditions.

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