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Annual variation in lung associated immunity and season dependent invasion of *Alternaria alternata* in lungs of Indian jungle bush quail, *Perdicula asiatica*

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Abstract

The purpose of this study was to evaluate annual variation in lung associated immune system (LAIS) along with general immunity of *Perdicula asiatica* an Indian tropical bird. Variation in immunological parameters such as size of bronchus associated lymphoid tissue (BALT) and non-BALT nodules, percent stimulation ratio (%SR) of isolated lung lymphocytes, total leukocyte count (TLC) and lymphocyte count (LC) was noted along with circulatory hormonal levels i.e. melatonin and testosterone for two consecutive years. Lowest immune status in terms of small BALT and non-BALT nodular size, %SR, TLC and LC was noted in the month of April. Considering the relation between annual variation of the peripheral hormones melatonin and testosterone and immune status of this bird, we observed an inverse relationship. It could be that high testosterone (an immunosuppressor) and low melatonin (an immunostimulator) levels during summer months are responsible for low immunity. While studying annual variation in LAIS we observed a fungal pathogen *Alternaria alternata* present in the lungs only during April suggesting that invasion occurred at a particular month of harvest (April). During the month of April low lung immune status was recorded, which could be responsible for such an invasion. This bird is a game bird and consumed as food by common rural people. If this fungus is being ingested along with the birds it may cause diseases like bronchitis, asthma, etc. in human being. Hence, gaming of this bird during summer month (April) should be avoided.

Keywords

Alternaria; LAIS; BALT nodule; melatonin; parasite; immunity

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Introduction

Perdicula asiatica is a seasonally breeding tropical bird with high food value for the rural people of plains in northern India. Many studies have been performed regarding reproductive aspects of this and other bird species (Haldar and Ghosh, 1990; Sudhakumari et al., 2001; Goymann et al., 2006) and seasonal immunity (Singh and Haldar, 2006). In north India at the vicinity of Varanasi (lat. 25°18′N; long. 83°1′E) the climatic factors show annual variation which significantly influence reproductive phases of the bird, *P. asiatica* (reproductively active period, RAP; June: photoperiod ∼14L : 10D; maximum and minimum temperature 37 ± 5°C and 26 ± 5°C; humidity ∼65% and a reproductively inactive period, RIP; January: photoperiod ∼11L : 13D; maximum and minimum temperature 15 ± 5°C and 6 ± 3°C; humidity ∼90%). Stressful conditions created by natural changes and pollutants from different sources as well as drastic changes in biotic and abiotic factors of ecosystem lead to seasonal fluctuation in immune function among individuals (Nelson and Drazen, 1999; Singh and Haldar, 2007). Furthermore, annual variation in general immunity and its relationship with pineal melatonin and gonadal hormone (testosterone) has already been established for this bird (Singh and Haldar, 2007).

The respiratory system is the first to encounter several pollutants and pathogens present in the environment. So a defense system is required to combat such agents. Our earlier study regarding a general survey of LAIS in *P. asiatica* confirms the existence of a well organized immune system in lungs with major components such as bronchus associated lymphoid tissue (BALT) nodules, non-BALT nodules, free lymphocytes and macrophages (Kharwar and Haldar, 2011a). BALT and non-BALT nodules are composed of T-cells and B-cells. BALT nodules are located at the junctions of primary and secondary bronchi while the non-BALT nodules are sparsely distributed throughout the lung (Reese et al., 2006). Since this bird is a seasonal breeder with annual variation in general immunity, variation in LAIS cannot be neglected. These birds feed on wheat grains and millet seeds dropped on the ground during harvest and while feeding those grains pathogens may enter via the oral or nasal cavities of the birds. After invasion of the respiratory or digestive system, the pathogens may cause diseases. However, no report till date is available regarding annual variation in LAIS of any wild species of birds.

It is evident that environmental factors such as photoperiod, temperature and humidity, food availability, social factors and stress originated from those do influence physiology of birds and circulatory levels of hormones (gonadal, adrenal steroid and melatonin level) which in turn affect the immune status (Singh and Haldar, 2005). Further *P. asiatica* is a wild bird living in fluctuating environmental conditions and hence encounters respiratory stress more than any poultry birds. Therefore, the objective of our present study was to check the annual variation of LAIS and invasion of any pathogen in the lungs of *P. asiatica* (an edible bird), thus opening a new site for zoonosis in humans as *P. asiatica* is a game bird being consumed by rural population.

Materials and methods

Experiments were conducted on healthy adult male *P. asiatica*, collected from the vicinity of the Varanasi (lat. 25°18′N; long. 83°1′E). Annual variations in climatic changes (day length, temperature and humidity) were recorded at Varanasi during the first week of every month. Average of maximum and minimum of the climatic parameters for two consecutive years which includes two major reproductive phases (RAP; June: photoperiod ∼14L : 10D; maximum and minimum temperature 37 ± 5°C and 26 ± 5°C; humidity ∼65% and RIP; January: photoperiod ∼11L : 13D; maximum and minimum temperature 15 ± 5°C and 6 ± 3°C; humidity ∼90%). The experiment was performed in accordance with institutional practice and within the framework of revised animals (Committee for the Purpose of Control and Supervision of Experiments on Animals; CPCSEA) Act of 2007 of Govt. of India on animal welfare.

Histological study

Six adult male birds (weight ≈ 45 mg) were sacrificed during the first week of every month of the year under complete anesthesia by injecting Nembutal (Sodium pentobarbital) around 3 hours after sunset (19:30-20:30; Kharwar and Haldar, 2011a, b). Lungs were infiltrated with Bouin's fluid *in situ* and then dissected out, cleaned and fixed again in Bouin's fluid by immersion overnight for routine histology (Kharwar and Haldar, 2011a, b). Transverse sections of entire lungs cut at 6 μm thickness were stained with Harris hematoxylin and eosin (1% alcoholic). Histological observation of lung tissue, i.e., size of the bronchus associated lymphoid tissue (BALT) nodule and non-BALT nodule was performed with the help of Filar ocular micrometer (WEBCON, India) to present the changes observed during twelve months of the year. Ten sections of entire lungs from each bird were randomly selected for morphometric analysis of twenty BALT and non-BALT nodules.

Gross examination of lung and identification of fungus

Careful examination of lung tissue was done to check all types of abnormalities, lesions or infection by pathogens, if any, throughout the year by performing routine histology. The observation of infection and identification of the fungus present in lungs of *P. asiatica* was carried out at the Department of Plant pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, India under supervision of Prof. Asha Sinha.

Immunological parameters

i) *% stimulation ratio of lung lymphocytes*. After sacrifice the lung tissue of six male birds was dissected out and cleaned from adherent tissues, washed with chilled PBS and then minced into small pieces in chilled RPMI-1640 with scissors in a sterile watch glass. It was then passed through a steel strainer of 400 meshes to remove

major lung tissue fragments and cell suspension was collected in a sterile centrifuge tube and washed twice with RPMI-1640 (Sigma-Aldrich Chemicals, St Louis, Missouri, USA). The red blood cells present in the cell suspension were lysed with cold ammonium chloride tris buffer [tris (hydroxymethyl) aminomethane, BDH, UK]; 0.5% tris buffer and 0.84% NH_4Cl mixed in 1 : 10 ratio and adjusted to pH 7.2. The procedure was repeated twice to exclude maximally red blood cells and other cells. The cell pellet was suspended in 2 ml of RPMI-1640 and filtered through 15 μm filters to get lymphocytes. The purity of the single cell suspension was monitored under inverted microscope and smear of cell suspension on glass slide was stained with Giemsa stain for more accuracy of purity. The cell viability was then checked with 1.0% Trypan blue exclusion method. Counting of isolated lymphocytes was performed with Neubauer's counting chamber (Spencer, USA), under Nikon Microscope. This single cell suspension of isolated lung lymphocytes (~95%) was adjusted to 1×10^6 cells/ml in RPMI-1640, containing sodium bicarbonate, antibiotics (Penicillin 100 IU/ml, streptomycin 100 μg/ml, gentamycin 100 μg/ml) and 10% foetal calf serum (Sigma, USA). For the study of blastogenic response the cell suspension was divided into aliquot of 2 ml each (10^6 cells/ml) and the control plates were cultured in the absence of mitogen whereas the test cultures were stimulated with mitogen Concanavalin A (Con A; Sigma-Aldrich Chemicals, St Louis, Missouri, USA) at the concentration of 5 μg/ml. The plates were incubated at 41°C under 5% CO_2 incubator (Hera Cell, Germany) for 72 h. Eighteen hours before harvesting, 1 μCi of tritiated thymidine [^{3}H] (BARC, India; specific activity 8.9 Ci/mM) was added to each culture plate. Culture plates were harvested after 72 h of incubation. Blastogenic response was measured in terms of [^{3}H] thymidine uptake against stimulation by Con A of the lung lymphocytes (Pauly and Sokal, 1972; Singh and Haldar, 2007a). The data was presented as stimulation ratio percentage (SR) which was calculated from count per minute (CPM) of lymphocytes isolated from lung using β counter (Beckman Coulter, USA).

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

ii) *Peripheral total leukocyte count/lymphocyte count*. Total leukocyte count (TLC) and lymphocyte count (LC) was done following the staining method of Singh and Haldar (2007a). In short, following sacrifice, trunk blood was collected in a sterile tube and later into a WBC pipette of Haemometer (Spencer, USA) and diluted 20 times in Turk's fluid (2.0 ml Glacial acetic acid, 0.1 g mercuric chloride, one drop aniline, and 0.2 g Gentian violet). The number of white blood cells was counted (no./mm^3) in a Neubauer's counting chamber (Spencer, USA), under Nikon Microscope. Thin film of blood was prepared and stained with Leishman's stain and differential leukocyte (lymphocyte) number was counted under oil immersion lens of Leitz MPV3 microscope. Lymphocyte count (no./mm^3) was determined from the total and differential leukocyte count by using the following formula:

$$\text{lymphocyte count} = \frac{\text{TLC} \times \text{lymphocyte percentage}}{100}.$$

Hormonal analysis

Trunk blood from sacrificed birds was taken in a heparinized tube and centrifuged at 1000× *g* at 4°C for 15 minutes to collect the plasma. Plasma was stored at −20°C for hormonal assay of melatonin and testosterone. For plasma melatonin, radioimmunoassay was done following the modified method of Rollag and Niswender (1976) as published by Singh and Haldar (2007a, b) and RIA kit from Immunochemical Corporation, Carson, USA was used for plasma testosterone assay. The validation of RIA was performed as described earlier (Sudhakumari et al., 2001). The sensitivity for melatonin RIA was 18-20 ng/ml and for testosterone RIA was 6 ng/ml. The intra and inter assay variation for melatonin was 9 and 15% and for testosterone 4.5 and 5.6% respectively. The recovery of testosterone and melatonin RIA was 95% and 92% respectively.

Statistical analysis

Statistical analysis of the data for BALT and non-BALT nodule size, immunological parameters, fungal spores and hyphae and hormonal level was performed with help of one-way ANOVA followed by Student Newman-Keuls' Multiple Range test. Results are expressed as mean ± SEM. The differences were considered significant with $P < 0.05$ and highly significant when $P < 0.01$ (Bruning and Knitz, 1977). Microsoft Excel program was used for statistical calculations and data presentation.

Results

The study was performed over two consecutive years. There was no significant difference in the data of both the years, hence we are presenting the data of one year only.

Histological study

Significant decrease in size of BALT and non-BALT nodules was noted during the month of April when compared with the rest of the months (BALT nodule: $F_{11,60} = 34.77$ and non-BALT nodule: $F_{11,60} = 253.70$; $P < 0.01$). A sharp decline in size of nodules was noted from January to April with minimum size in the month of April (BALT nodule: 1.42×10^2 μm and non-BALT nodule: 1.15×10^2 μm). From May to December an increase in nodular size was noted with maximum size in the month of December (BALT nodule: 3.3×10^2 μm and non-BALT nodule: 3.22×10^2 μm) (figs 1b, 1c, 4a).

Gross examination of lung tissue and identification of fungus

Gross examination of lung histology presented no lesions or abnormalities in lung throughout the year except in the month of April. The characteristics of the fungal elements, i.e., spores and hyphae were identified in lungs of birds during April

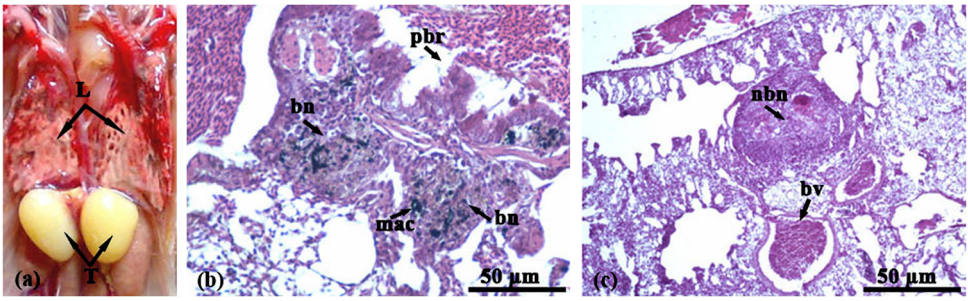

Figure 1. (a) Photograph showing comparative anatomy of lung of *P. asiatica* (L) and testes (T) during reproductively active phase (RAP; April). Photomicrographs showing (b) BALT nodule and (c) non-BALT nodule in the transverse sections of lung of *P. asiatica* during reproductively active phase (RAP; April); bn: BALT nodule, pbr: primary bronchus, mac: macrophage, nbn: non-BALT nodule, bv: blood vessel. This figure is published in colour in the online version.

(temperature ~35°; photoperiod ~12.45; humidity ~40.31) as *Alternaria alternata* (figs 2, 3) under guidance of experts in the Department of plant pathology, Institute of Agriculture, Banaras Hindu University, Varanasi, India. Interestingly no traces of other fungal varieties and or of *A. alternata* were observed in lung tissue of *P. asiatica* during the rest of the year (fig. 4b).

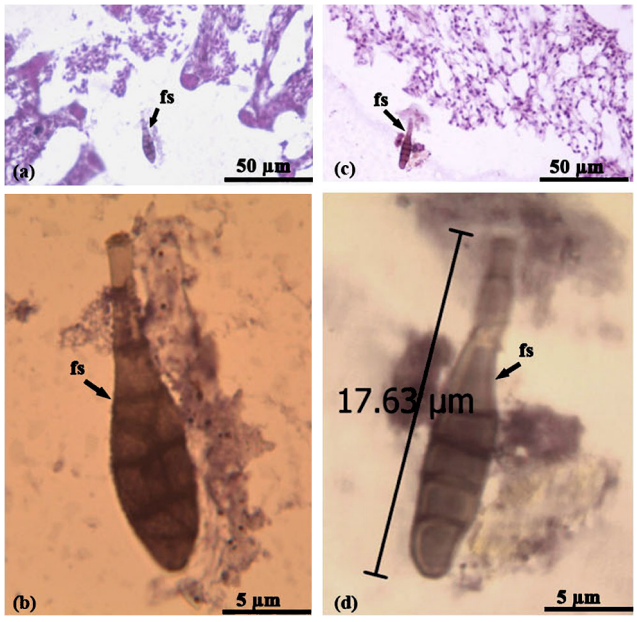

Figure 2. Photomicrographs showing spores of *A. alternata* under low magnification (a, c) and under high magnification (b, d) in transverse section of lung of *P. asiatica* during April (RAP). This figure is published in colour in the online version.

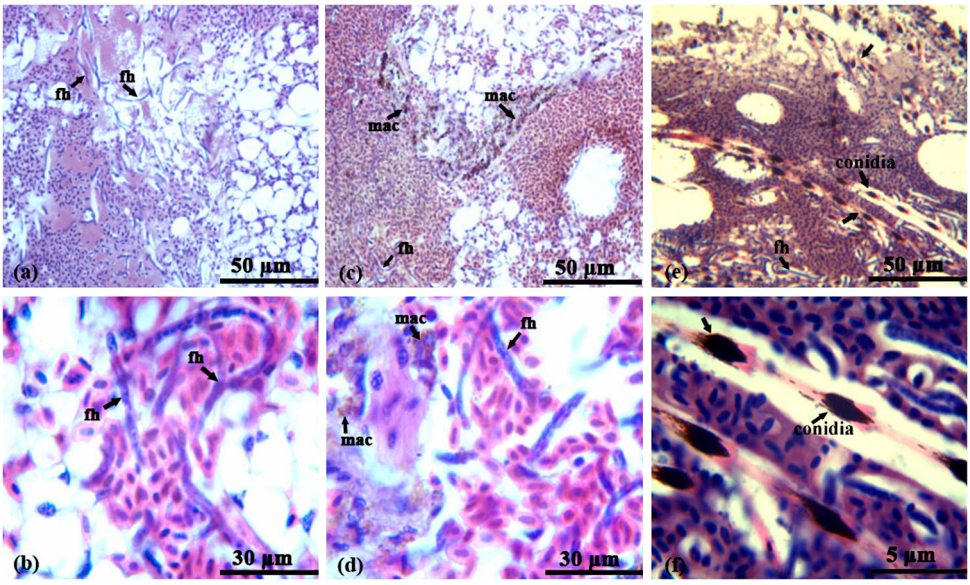

Figure 3. Photomicrographs of lungs showing (a) fungal hyphae of *A. alternata* scattered throughout the lung tissue, (b) enlarged view of fungal hyphae scattered throughout the lung tissue, (c) macrophages and fungal hyphae scattered in the lung tissue, (d) enlarged view of macrophages and fungal hyphae scattered in the lung tissue, (e) conidia moving in parabronchial space in lung tissue, and (f) enlarged view of conidia moving in parabronchial space in lung tissue *P. asiatica* (fh: fungal hyphae, mac: macrophage) during April. This figure is published in colour in the online version.

Variation in lung associated and general immunological parameters

Significant difference in %SR was noted from November to February when compared with month of April ($F_{11,60} = 34.44$; $P < 0.01$). A gradual decline in %SR was noted from January to April with minimum value in the month of April (201.11%). From May to December an increase in %SR was noted with maximum value in the month of December (278.6%) (table 1).

Significant decrease in TLC was noted during month of April when compared with rest of the months ($F_{11,60} = 89.15$; $P < 0.01$). A sharp decline in TLC was noted from January to April with the minimum value in the month of April (33 467). From May to December an increase in TLC was noted with maximum value in the month of December (69 800) (table 1).

Significant decrease in LC from peripheral blood was noted during month of April when compared with rest of the months ($F_{11,60} = 52.17$; $P < 0.01$). A sharp decline in LC was noted from January to April with the minimum value in the month of April (7760). From May to December an increase in LC was noted with maximum value in the month of December (19 400) (table 1).

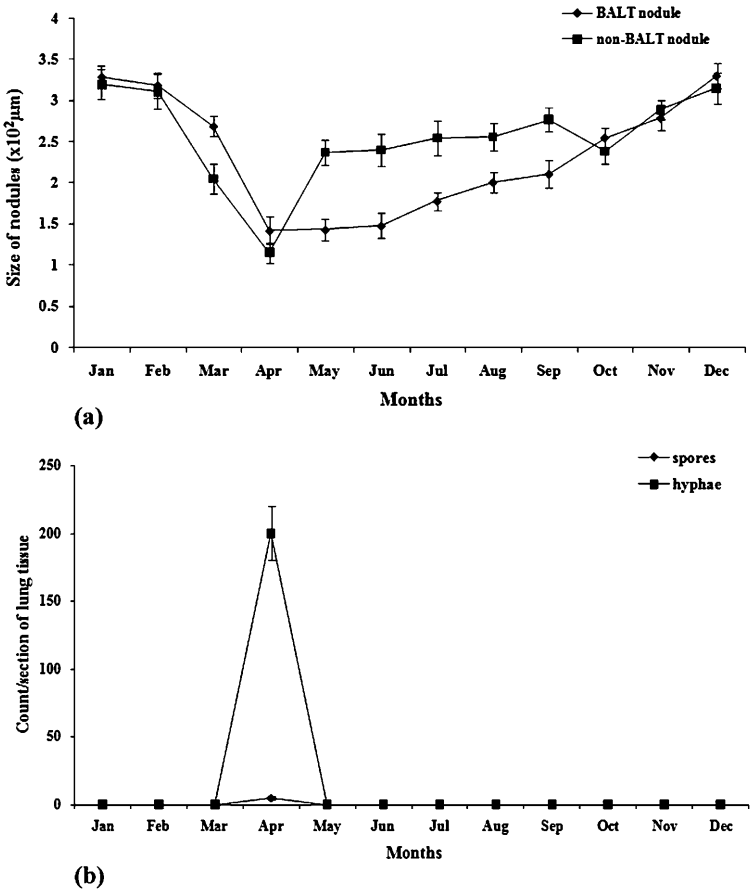

Figure 4. (a) Annual variation in size (μm) of BALT and non-BALT nodules in lung of *Perdicula asiatica* (mean ± SEM); (b) Annual variation in number of spores and fungal hyphae in lung of *P. asiatica* (mean ± SEM).

Variation in hormonal level

Significant decrease in peripheral melatonin level was noted during the month of April when compared with the rest of the months ($F_{11,60} = 328.24$; $P < 0.01$). A sharp decline in circulatory melatonin level was noted from January to April with the minimum value in the month of April (32.6 ± 7.37 pg/ml). From May to December an increase in peripheral melatonin level was noted with the maximum value in the month of December (193.6 ± 5.98 pg/ml) (table 1).

Significant increase in peripheral testosterone level was noted during month of April when compared with rest of the months ($F_{11,60} = 540.03$; $P < 0.01$). A sharp increase in testosterone level was noted from January to April with the maximum value in the month of April (59.25 ± 1.47 ng/ml). From May to December a sharp decrease in testosterone level was noted with the minimum value in the month of December (9.51 ± 1.71 ng/ml) (table 1).

Table 1.
Annual variation in immunological parameters and hormonal level.

Months	Parameters				
	%SR	TLC (no./mm^3)	LC (no./mm^3)	Testosterone (ng/ml)	Melatonin (pg/ml)
Jan.	273.40 ± 11.67	65 790 ± 1825	18 360 ± 769.9	10.74 ± 1.27	129.9 ± 8.10
Feb.	268.10 ± 10.5	64 945 ± 3083	18 300 ± 890.79	11.86 ± 1.15	82.4 ± 7.31
Mar.	207.50 ± 12.16	42 400 ± 2450	10 800 ± 1315.91	13.83 ± 1.12	40.43 ± 6.13
Apr.	**201.11[a] ± 18.42**	**33 467** ** **± 1550**	**7760** ** **± 1007.05**	**59.25** ** **± 1.47**	**32.6** ** **± 7.37**
May	203.56 ± 13.71	37 250 ± 2220	9685 ± 1493.6	23.21 ± 1.70	53.7 ± 9.05
June	205.18 ± 9.31	39 935 ± 2972	10 872 ± 1544.4	20.37 ± 1.31	59.1 ± 7.90
July	206.60 ± 12.27	42 160 ± 4359	11 945 ± 1519.4	19.24 ± 1.26	73.58 ± 7.79
Aug.	207.43 ± 10.15	44 180 ± 2087	12 400 ± 1212.01	18.59 ± 1.67	90.8 ± 4.87
Sep.	209.19 ± 11.12	48 700 ± 1737	13 580 ± 1079.7	18.11 ± 1.50	118.8 ± 5.26
Oct.	219.65 ± 14.47	54 280 ± 4704	15 150 ± 1150.8	12.35 ± 1.16	159.23 ± 6.64
Nov.	267.30 ± 17.18	57 000 ± 4094	15 823 ± 1620.5	11.22 ± 1.42	162.2 ± 7.62
Dec.	278.60 ± 16.26	69 800 ± 4352	19 400 ± 1323.2	9.51 ± 1.71	193.6 ± 5.98

Data are presented as mean ± SEM. Number of birds was 6.
** $P < 0.01$ immunological parameters and hormonal level in April *vs* rest of the months.
[a] $P < 0.01$ %SR in April *vs* Nov-Feb.

Discussion

For the first time, we present an account of annual variation in LAIS of *Perdicula asiatica* and its relation with hormonal variation in melatonin and testosterone throughout the year and the occurrence of pathogenicity in the lungs by *A. alternata*. The study was performed for two consecutive years with the birds under natural environmental conditions so that an impact of general immune status and LAIS on the presence of pathogens could be suggested. The pathogen invasion is maximum when there is weak immune status of the host because the host provides required microenvironment and food for the pathogen.

While accounting LAIS, we found the annual changes in size of BALT and non-BALT nodules, TLC, LC and %SR of isolated lung lymphocytes which presented an apparent variation along with general immune status of the birds (Singh and Haldar, 2007). These immune parameters which account for LAIS as well as general immunity showed changes parallel to each other and directly proportional to circulatory melatonin and inversely to testosterone as noted earlier (Singh and Haldar, 2007; Kharwar and Haldar, 2011b). Increase in size of BALT and non-BALT nodules, TLC, LC, %SR of lung isolated lymphocytes and plasma melatonin concentration was reported in December when days were short (∼11L : 13D), which is the reproductively inactive state of this bird (Kharwar and Haldar, 2011b). The maximum decrease in above mentioned immunological parameters and low plasma melatonin concentration was noted in April when days were long (∼14L : 10D), proving the inverse relationship between melatonin and testosterone (Singh and Haldar, 2007).

Thus photoperiod and temperature probably play a role in the regulation of the parameters of LAIS as it does for general immunity (Panshikar and Haldar, 2009; Kharwar and Haldar, 2011b). This fact was supported by reports for broiler chickens under manipulated photoperiodic exposures (Kliger et al., 2000) and latitudinal variation in immune defense of white crowned sparrow (Owen-Ashley et al., 2008).

It is suggested that winter associated stressors (biotic and abiotic factors) counteract short day enhancement of immune function by melatonin (Demas and Nelson, 1996; Kumar et al., 2007). Short photoperiod of winter enhanced circulatory melatonin level and this high melatonin level coincided with high lung associated immune status as depicted by bigger size of BALT and non-BALT nodules and increased %SR and general immunity in terms of TLC and LC (Kharwar and Haldar, 2011b). Thus, the increasing trend of melatonin concentration in serum during the winter suppressed the strong effect of winter stressors and induced the lung associated immune parameters and helped the bird to remain healthy and fit in order to combat winter born respiratory diseases (e.g. avian flue). During long photoperiod (summer days), higher gonadal steroids in circulation is responsible for reproductive activity in this birds and thereby decreases the immune status as steroid suppresses immunity in general (Schuurs and Verheul, 1990; Singh and Haldar, 2005; Weil et al., 2006). Thus, we may suggest that variation in peripheral melatonin by natural light (Gwinner and Scheuerlein, 1998) conditions acts as a bolster to the immune function, on one hand, and suppressed the gonadal activity in winter, on the other hand, to help the individuals to have more energy to fight with seasonal stressors (Nelson and Drazen, 1999). Surprisingly, in the Red Knot (*Calidris canutus*), Buehler et al. (2009) found no such correlation and suggested that immunocompromise should be correlated with the severity of the environment rather than the time of year. Therefore, variation in lung associated immune status noted throughout the year in *P. asiatica* of tropical zone might be responsible for adaptations that have evolved to decrease the odds of respiratory system in changing environmental conditions.

Annual gross morphology of lung tissue of *P. asiatica* suggests neither any lesion nor any wound (fig. 1a) reflecting that birds were healthy without any severe infection in lungs. Testicular size was maximum during summer as reported earlier (Sudhakumari et al., 2001) for this bird which is a long day breeder. Furthermore fig. 1a shows *in situ* size of the testes during April. From histological observation, we found fungal hyphae and spores of *Alternaria alternata* only during summer month of April. Presence of macrophages throughout the lung was also noted (fig. 3b) suggesting that there was an invasion of a foreign agent in the lungs of *P. asiatica*. It is to be recalled that this is the period of a year when maximum cases of bird flu is reported in the past. Our result reveals that the general as well as LAIS was low during April due to high level of testosterone (immunosupressor) as it is RAP and low melatonin (immunostimulator) level due to long days of summer. This condition might have helped the fungal growth to be dominant in April and recessive during rest of the year when melatonin is high.

Perdicula asiatica is a game bird and local people of northern India use it as a source of food. This bird is available during summer season when crops are harvested. Birds feed on the crop grains fallen on the ground and this becomes the cause of transmission of fungal spores from soil to birds and later may be from a wild bird to human. *Alternaria* species are also ubiquitous in nature, common in corn and agricultural commodities. Most mycotic infections affect the respiratory or alimentary tracts of birds. In poultry, ingestion of *Alternaria* species is known to cause subcutaneous, epicardial and intestinal hemorrhage and death from tenuazonic acid (Hoerr, 1991), a potent mycotoxin (Miller, 1994). *Alternaria* produces large spores having sizes between 20-200 microns in length and 7-18 microns in width, suggesting that the spores from this fungus are deposited in the nose, mouth and upper respiratory tract (Wilken-Jensen and Gravesen, 1984). This mold is an important source of allergens for mold allergic patients. This fungus, once considered non-pathogenic to humans, has now been shown to cause invasive disease in both presumably healthy and immunocompromised hosts. *Alternaria* also causes invasive infections in both mucosal and visceral tissue. Host defenses against *Alternaria* have not been carefully studied. The clinical reports suggest, however, that cell-mediated immunity or phagocytic cells may provide protection against infection due to this organism (Azar et al., 1975; Farmer and Komorowski, 1976; Mardh and Hallberg, 1978; Loveless et al., 1981; Goodpasture et al., 1983). We also know that short photoperiod increases circulatory melatonin level (Panshikar and Haldar, 2009; Kharwar and Haldar, 2011c) and melatonin in turn enhances cell-mediated immunity and phagocytic activity in lung. So, higher peripheral melatonin might be maintaining the lung immune status of quails during all other months of the year thereby reducing the fungal infection. Initial decline in melatonin level during April might have provided the opportunity of parasitic invasion in lung to multiply.

Thus, the occurrence of *A. alternata* in lung of *P. asiatica* expands the spectrum of opportunistic infections in wild birds suggesting the importance of season dependent lung immune status in host defense against this organism. In order to prevent zoonosis, gaming of *P. asiatica* for food may be avoided in summer months.

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