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Seasonal Phenotypic Flexibility And Histomorphometry Of Thyroid Gland Of Redheaded Bunting

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Abstract

The present study was carried out to demonstrate a comparative account of distinctive histological features of the thyroid gland in male and female redheaded buntings (*Emberiza bruniceps*) maintained under natural and artificial laboratory conditions, during pre-breeding and breeding seasons, with respect to their physiological indices. 48 adult birds were procured during the over-wintering season in India and divided into two groups each of 24 birds (12 birds/ sex). One set of birds was housed in an outdoor aviary, while the other set was transferred to an indoor aviary. In both aviaries, six birds/ sex were used for collecting data on physiological indices and histomorphometry. Gonadosomatic index (GSI) and fatness index were significantly different between NDL/ artificial conditions, pre-breeding/ breeding seasons, and male/ female birds. GSI was also influenced by the condition of the study. Histologically, the thyroid gland was observed to be enclosed by a thin capsule that showed sex-dependent differences. Follicular area and follicular density were significantly different between pre-breeding and breeding season. The cross-sectional area of the thyroid gland was significantly affected by the interaction between condition x sex and season x sex. These results suggest that the surrounding condition, life history state (LHS), and sex of the birds play a key determining role in regulating the seasonal phenotypic plasticity at the level of physiology and organ plasticity. Histomorphometric changes in the thyroid gland are suggestive of its season-dependent differential activity.

Keywords: Fatness index, histomorphometry, redheaded bunting, seasonality, thyroid follicle, thyroid gland, gonado-somatic index

1.0. Introduction

The role of photoperiod and temperature information in translating migratory events has been previously studied, highlighting the involvement of thyroid hormones in the physiological timing of migration in redheaded buntings (Chandola & Pathak, 1980; reviewed by Nair et al., 1994). Studies have shown that the thyroid hormones, thyroxine (T4) and triiodothyronine (T3), are involved in the development of premigratory disposition, which includes excessive fat deposition and nocturnal restlessness (*Zugunruhe*) in captivity (Pant & Chandola-Saklani, 1993). The role of the thyroid glands has also been demonstrated by its ablation. Thyroid ablation experiments have demonstrated that the removal of the thyroid glands completely prevented the development of premigratory behaviors, whereas, the replacement therapy with T4 or T3 restored both fat deposition and *Zugunruhe* in buntings (Pant & Chandola-Saklani, 1993; Pathak & Chandola, 1982), thereby indicating the importance of these hormones in migratory physiology. Additionally, the conversion of T4 to T3 is essential for these processes, as blocking this conversion suppresses both fattening and *Zugunruhe*. However, interestingly, the conversion of T4 to T3 did not affect feather regeneration in buntings, hence, suggesting that different physiological processes are regulated by these hormones in distinct ways (Pati & Pathak, 1986). Research on redheaded bunting has also investigated the effects of thyroidectomy and replacement therapy with L-T4 on liver and plasma biochemical constituents. This study found that thyroidectomy significantly elevated levels of plasma glucose, protein, cholesterol, and diglyceride, while depressing hepatic free fatty acid levels (Singh et al., 1992). The role of the thyroid gland in photorefractoriness of seasonal breeders has also been studied in past, for example, in redheaded buntings, thyroidectomy accelerated the absolute photorefratoriness, partially inhibited gonadal recrudescence, and completely inhibited photoinduced gain in body mass (Thapliyal & Lal, 1984). These research outcomes highlighting the differential functioning of the thyroid glands shed light upon the possibilities of different morphology.

The thyroid gland is composed of many follicles, whose size can vary depending on the bird's physiological state. Increasing daylength has a stimulatory effect on the conversion of T4 to active T3 (Yasuo et al. 2005), During periods of high thyroid activity, the follicles tend to be larger and more active (House sparrow; Kendeigh & Wallin, 1966), with a higher number of epithelial cells and increased colloid production. This enlargement is associated with increased production of thyroid hormones, which play a crucial role in preparing the bird for migration (Sharma et al., 2018). Conversely, during periods of low thyroid activity; after the migration period or summers, the thyroid gland returns to its normal size, and the follicles become less active. This suggests that seasonal variations in the histomorphometry of the thyroid gland and thyroid follicles could be observed in a seasonally breeding bird such as redheaded bunting.

The thyroid gland, a pivotal component of the endocrine system, plays a crucial role in regulating metabolism, growth, and development across vertebrates. Its function is particularly significant in birds, where it influences various physiological processes, including reproduction, migration, and molting (McNabb, 2007; Yasuo et al., 2005; Yoshimura, 2013). Molting is critical for maintaining feather integrity and aerodynamic efficiency in birds (Dawson et al., 2001) and is energetically costly and often coincides with periods of reduced reproductive activity and increased energy storage (Murphy, 1996). The timing and extent of molting are influenced by thyroid hormones, which promote the growth of new feathers and the shedding of old ones (Payne, 1972). The histomorphometry of the thyroid gland, which involves the quantitative assessment of its structural components, provides valuable insights into its functional status and its adaptation to different physiological demands in a seasonally breeding bird having distinct Life history states (LHSs).

The redheaded bunting is a long-distance migratory bird that breeds in Central Asia and overwinters in the Indian subcontinent (Ali & Ripley, 1999). It exhibits distinct seasonal changes in physiology and behavior, driven by photoperiodic cues (Sharma et al., 2018). Before spring migration, the birds undergo a period of pre-migratory fattening, during which they accumulate substantial energy reserves to fuel their long-distance migration (Rani et al., 2005). These seasonal changes are likely mediated by alterations in thyroid activity, which in turn may be reflected in the histomorphometry of the thyroid gland. Migration is an energetically demanding process that requires precise coordination of various physiological systems, including the endocrine system, to ensure the successful completion of the journey (Gwinner, 1996). Thus, the thyroid gland, through its secretion of thyroid hormones, plays a critical role in modulating behavior, energy metabolism, thermoregulation, and overall physiological preparedness for migration (Nair et al., 1994; Perez et al., 2016; Singh et al., 1992;). Additionally, the thyroid gland's activity is closely linked to reproductive physiology, influencing gonadal development and function (Chandola & Pathak, 1980; Yoshimura, 2013). Therefore, understanding the histomorphometric changes in the thyroid gland of migratory birds like the redheaded bunting can provide insights into the endocrine mechanisms underlying their complex life history traits.

Histomorphometry of the thyroid gland involves the measurement of various structural parameters, such as capsule thickness, glandular area, follicular density, follicular area, and height of epithelial cells secreting the colloid in the lumen of follicles (Parchami & Dehkordi, 2012; Mattsson et al., 2019; Ozkanlar et al., 2021). These parameters are indicative of the gland's functional state. For instance, an increase in the number and size of active follicles may suggest heightened thyroid activity, which could be associated with increased metabolic demands during migration or reproduction (Beyzai & Adibmoradi, 2011). Conversely, a reduction in these parameters might indicate a period of reduced activity or quiescence. The histomorphometry of thyroid gland has been studied in various avian species, revealing species-specific adaptations to different environmental and physiological conditions. For example, in domestic fowl (*Gallus gallus domesticus*), thyroid activity increases during periods of rapid growth and high metabolic demand (Scanes, 2009). In migratory species like the European robin (*Erithacus rubecula*), thyroid gland activity peaks during the pre-migratory phase, coinciding with *Zugunruhe* and increased fat deposition (Farner, 1950). These studies highlight the importance of thyroid hormones in mediating physiological changes associated with migration and reproduction. However, there is a paucity of data on the histomorphometry of the thyroid gland in small passerine migrants like the redheaded bunting, particularly in relation to their breeding strategies.

In the present study, we measured the capsule thickness of the thyroid gland, the cross-sectional area of the thyroid gland, the number of follicles in a given area (follicular density), and the area of large active follicles. These measurements were correlated with the gonado-somatic index (GSI) (Singh et al., 2022) and fatness index (Koivula et al., 1995; Nord et al., 2011), which are indicators of reproductive status and energy reserves (body condition), respectively. GSI, calculated as the ratio of gonad weight to body weight, is a widely used parameter to assess reproductive activity in birds. It reflects the investment of energy into gonadal development, which is crucial for successful breeding (Wingfield & Farner, 1993). The fatness index, on the other hand, provides an estimate of the bird's energy reserves, which are critical for sustaining long migratory flights (Blem, 1990; Nord et al., 2011) and other physiological energy-demanding processes. Both indices are perhaps influenced by thyroid activity, as thyroid hormones are known to modulate lipid metabolism and reproductive function (Decuypere et al., 2005). Therefore, examining the relationship between thyroid histomorphometry and these indices can shed light on the endocrine regulation of LHSs in redheaded buntings.

The histomorphometric analysis of the thyroid gland in redheaded buntings is expected to reveal significant variations in glandular structure in relation to the birds' reproductive and migratory status. For instance, during the breeding season, when metabolic demands are high, the thyroid gland may exhibit increased activity, as indicated by a greater number of large active follicles and a thicker glandular capsule. Conversely, during the non-breeding season or periods of migration, the gland may show signs of reduced activity, with fewer and smaller follicles. These changes are likely to be correlated with variations in the GSI and fatness index, reflecting the interplay between thyroid activity, reproductive investment, and energy storage.

Therefore, this study focuses on the histomorphometric analysis of the thyroid gland in the redheaded bunting (*Emberiza bruniceps*), a small passerine migratory finch, to understand its physiological adaptations in relation to pre-breeding and breeding phases that are temporally aligned before and after the spring migration, respectively. By examining the thyroid gland's structural parameters in relation to the GSI and fatness index, this study aims to elucidate the role of the thyroid gland in coordinating the temporal physiological demands and aims to contribute to understanding the complex life history traits of migratory birds. Insights from the histomorphometric analysis of the thyroid gland in redheaded buntings will have broader implications for avian physiology and conservation. By elucidating the endocrine mechanisms underlying migration and reproduction, this study contributes to a deeper understanding of the physiological adaptations that enable migratory birds to cope with changing environmental conditions and meet reproductive demands. Furthermore, the findings of this study may inform conservation strategies aimed at protecting migratory bird populations and their habitats.

2.0. Materials and methods

2.1. Animal and maintenance

Redheaded buntings were captured during their overwintering period in India. It is a migratory bird that arrives in India after autumn migration and overwinters from August to March/April, while it breeds in Central Asia from May to June (Ali & Ripley, 1999). For the study, both male and female redheaded buntings were mist-netted from the wild during mid-February from the outskirts of Lucknow (26°55′N, 80°59′E), India. During this time, the average daylength was ~11.25 h, while temperature and relative humidity in nature were 17.14 ± 1.97 °C and 73.29 ± 7.72 %, respectively. We acclimatized the procured birds to captive conditions in an outdoor aviary (size: = 3.0 x 2.5 x 2.5 m). Tetracycline hydrochloride antibiotic (Hoechst Roussel Vet Pvt. Ltd) was given in water during the first five days of acclimation period. Seeds of *Setaria italica* (Fox tail millet) and water were provided *ad-libitum*. A special supplementary feed (Singh et al., 2010) was provided twice per week to all the birds. The acclimatization period in the aviary was ~2 weeks.

2.2. Study protocol

The study was performed after the ethical approval from the Institutional Animal Ethics Committee (IAEC) of the Department of Zoology, University of Lucknow, India (protocol no.: LU/ZOOL/IAEC/01/21/02). We randomly selected 48 birds and distributed them in two sets of 24 birds each; each set having 12 male and 12 female birds. The first set of birds was used for study under NDL condition (natural daylength and temperature condition) in an outdoor aviary and the second set of birds was used for the study under artificial condition in indoor chronocublicle (2.2 x 1.8 x 2.8 m) located in the basement facility of the laboratory, devoid of any natural cues. Under both conditions, physiological indices (GSI and fatness index) were measured and histomorphometric analysis of the thyroid gland was done during pre-breeding and breeding season (6 birds/ sex/ season). From NDL, physiological data was recorded and thyroid glands were harvested during March (Vernal Equinox) for pre-breeding phase and during June (Summer Solstice) for breeding phase.

For the study under artificial condition, by the end of February the acclimatized birds were transferred to the basement facility and kept under 8L:16D photoperiod until the last week of October, when the study under artificial conditions began. Thereafter, we started providing them simulatory daylengths for a daily change in lights onset and offset with reference to the photoperiod (sunrise to sunset) experienced by the birds in NDL from mid-February through Vernal Equinox until Summer Solstice in June, using electronic timers. It is to be noted here that, the birds under NDL received transitions in daylight intensity (sine-wave), while the birds under artificial set-up received constant light intensity (square-wave) throughout the photophase of the light-dark cycle. Under both conditions, body mass and gonadal weight were recorded to calculate to GSI, while tarsus length measurement and body mass were used to calculate the fatness index. The study parameters taken into account were recorded during midday, around the commencement of the Vernal Equinox and Summer Solstice timepoints (Fig. 1).

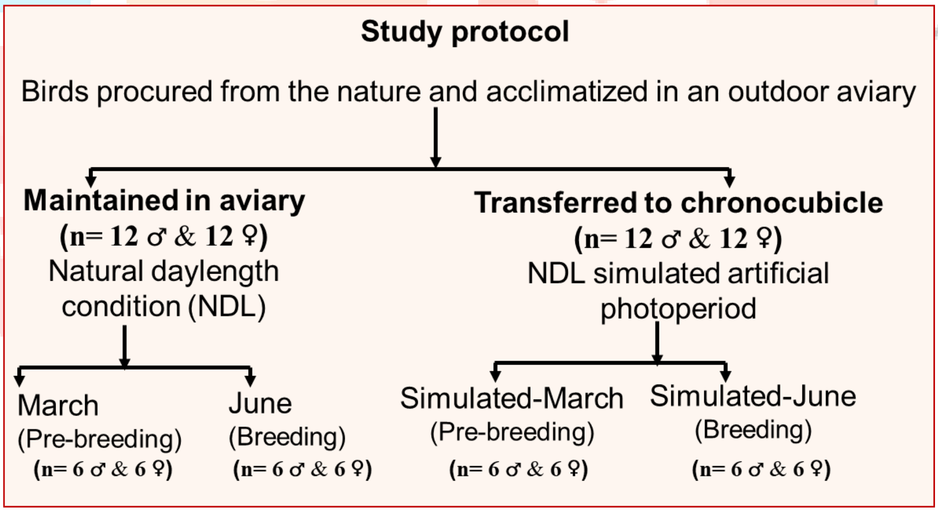

Figure 1: Study protocol showing distribution of male and female redheaded buntings in pre-breeding and breeding season under natural daylength and artificial conditions. During March and Simulated-March, data was collected on Vernal Equinox and during June and Simulated-June, data was collected on Summer Solstice.

2.3. Data collection

2.3.a. *Physiology*

For calculating the GSI of the birds using the formula: GSI = (testes weight or ovary weight/body weight) x 100 (Singh et al., 2022), we recorded their body mass using a top pan balance (Shimadzu ELB 300) having an accuracy of 0.01 g, while their paired testes weight and left ovary weight (right ovary being rudimentary) was recorded using an electronic balance (Sartorius BT 214 D) having an accuracy of 0.1 mg. After weighing, these organs were deep frozen at -80°C. We also recorded the length of the right tarsus using a digital calliper (following ESF manual; Bairlein, 1995) having an accuracy of 0.01 mm, to calculate the fatness index of the birds using the formula: fatness index = (1000 x body mass)/ tarsus length^3 (Koivula et al., 1995; Nord et al., 2011).

2.3.b. *Histomorphometry*

To study endocrine regulation of pre-breeding and breeding LHS in redheaded buntings, we chose to perform and analyze the histomorphometry of the thyroid gland. For this, the thyroid glands were harvested by decapitating the birds at midday on the day of Vernal Equinox and Summer Solstice under NDL condition and their simulated time points under the artificial condition. The harvested thyroid glands were post-fixed for overnight at 4°C in fixative (Majumdar et al., 2015). Following fixation, cryoprotection was done by passing the thyroid glands through 10%, 20%, and 30% sucrose solution until they sank in each grade. Embedding in 15% Polyvinylpyrrolidone (PVP) (HiMedia Laboratories Pvt. Ltd., Mumbai) solution was done post cryoprotection. Cryosectioning was done using cryostat (CM 1850 Leica, Germany) set at -25°C, at 6 µm thickness (Paul et al., 2011) in cross-sections. The sections were directly taken onto a subbed slide and were left for drying at room temperature for overnight before staining.

Haematoxylin-Eosin (H-E) staining was done to stain the sections for histomorphometric analysis. Briefly, the sections were first cleared in xylene for 3 mins followed by rehydration in descending grades of alcohol (100%, 90%, and 70%; 1 min each). Thereafter, the sections were washed in distilled water (1 min). Hematoxylin (Qualigens, Thermo Fisher Scientific India Pvt. Ltd., India) staining was done for 1 min. Afterward, if overstained, the sections were treated with acid water for 3-5 seconds (1 dip); if not, they were transferred to distilled water for 1 min and then to tap water for 2 mins. Tap water is rich in ions and thus serves as a bluing agent. Ascending grade 70% and 90% alcohol treatment was given for 2 mins each followed by eosin staining (Qualigens, Thermo Fisher Scientific India Pvt. Ltd., India) for 45 seconds. After staining, acid alcohol treatment was given if the sections were over-stained. Again, the sections were subjected to 90% alcohol followed by 100% for 2 mins each. For 30 seconds, the sections were now air dried before being subjected to two changes of xylene, each for 5 minutes to clear the sections. Finally, the sections were mounted in DPX (Merck Specialities Pvt. Ltd., Mumbai, India) and were dried at room temperature for at least 72 hours before capturing images using a light microscope (Leica DM 300).

A digital camera (Leica DFC-420C) attached to the light microscope, was used to capture images via Leica Application Suite software version 4.12. For imaging, the microscope was set at 100x magnification (ocular = 10x; objective = 10x) with the scale set at 200µm. All the captured images had a resolution of 2560 x 1920 pixels and during measurements, the scale was set at 2.0588 pixels/ micrometer with the help of the 'set scale' tool in the analyze drop-down menu. Images of 5 thyroid sections per individual, preferably taken from the widest part of the gland, from all 8 groups (n = 6/ group) were captured. In total 30 images per group and overall, 240 images from the entire study population were considered for the analysis purpose.

Using ImageJ software, US National Institutes of Health, Bethesda, MD, USA (Schneider, 2012), from the cross-sections of thyroid gland, we recorded eight measurements (see Chaves et al., 2018) for the capsule thickness at different regions of the periphery from each image using the line tool. Similarly, we measured the area of eight large and active colloid-filled follicles from each section, and thus 40 measurements of the capsule thickness/ individual and area of 40 follicles/ individual were measured using the 'polygon selection' tool. The gland's cross-sectional area was also measured using the polygon selection tool, and 30 measurements were recorded for each group. Thereafter, we measured the follicle density in thyroid sections by measuring the number of follicles lying in the 221 x 221 µm^2 area of the cross-section. A similar method has been used to calculate the follicular density by Sonne et al. (2010) in a study on Glaucous gulls (*Larus hyperboreus*). While taking measurements, markings were done to avoid repetition of the same spot on the capsule or the same follicle. Mean value of every bird was calculated by averaging the individual values obtained per image. Mean (± SEM) of each group was calculated by averaging the individual mean obtained earlier.

2.4. Statistics

We performed Generalized Linear Model (GzLM) analysis on our data using IBM SPSS ver. 20 software, to test the effect of condition (NDL/ artificial), season (pre-breeding/ breeding season), sex (male/ female), and their two-way and three-way interactions on physiological indices and histological components. Further, irrespective of the housing condition, to study the relationship between the parameters under consideration in male and in female birds, Spearman correlation analysis was performed using GraphPad Prism ver. 8.0.2, and the result for the same was interpreted through a correlation matrix. Bar graphs with scattered dot plots were designed using GraphPad Prism software version 8.0.2, Graphpad Software, Inc.

3.0. Results

The thyroid glands were located as small oval reddish-brown structures in the thoracic inlet (Fig. 2), caudally to the crop, one on either side of the trachea in proximity to the common carotid artery and jugular vein. Histological examination showed that the thyroid gland was composed of numerous follicles of varying sizes and the follicles were either colloid-filled, empty or had vacuolated colloid, and the follicles were surrounded by a single layer of epithelial cell whose shape ranged from cuboidal to flattened. Each cross-section of the gland was surrounded by a collagenous capsule. Numerous blood vessels supplying blood to the thyroid gland were observed in the histological examination. In some of the sections, the parathyroid gland was observed if it was harvested along with the main thyroid gland, embedded in adipose tissue as shown in Fig. 3.

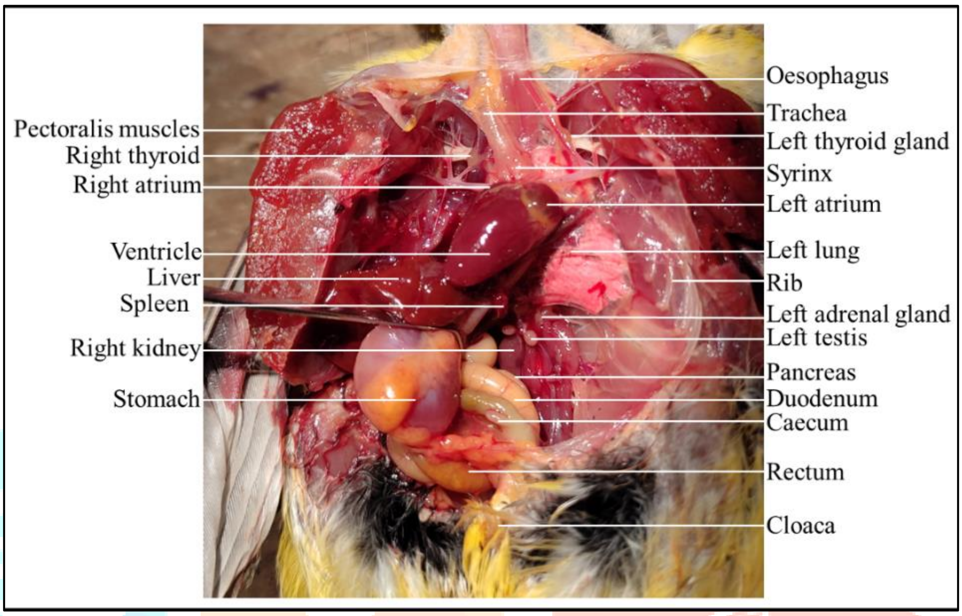

Figure 2: Anatomy of a male redheaded bunting showing location of different organs in the thoracic and abdominal cavity. Note the position of thyroid glands in the upper thoracic region.

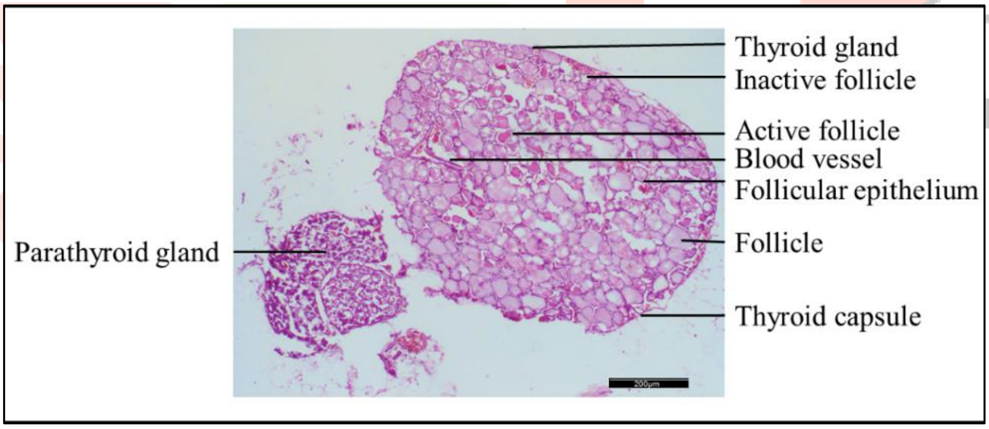

Figure 3: Histology of thyroid and parathyroid gland of redheaded bunting showing distribution of follicles. Scale bar: 200 µm.

3.1. Physiological indices

In redheaded buntings, GSI (Fig. 4i) was significantly affected by the conditions, seasons, sex of the bird, and interaction between condition and sex, and season and sex ($P < 0.05$; Table 1), however, it was not affected by the interaction between condition and season, and the three-way interaction ($P > 0.05$; Table 1). Whereas, the fatness index (Fig. 4ii) was significantly affected by the main effects of season and sex only ($P < 0.05$; Table 1). The condition of the study and other two-way and three-way interactions did not affect the fatness index of redheaded buntings ($P > 0.05$; Table 1). The results of the sequential Bonferroni post-hoc test are shown by symbols in Fig. 4.

3.2. Histomorphometric analysis

Conditions, seasons, and sex had no direct effect on the cross-sectional area (Fig. 5i-ii) of the thyroid gland of redheaded buntings ($P > 0.05$; Fig. 5ii; Table 1), while the interaction between condition and sex as well as sex and season significantly affected the cross-sectional area of the thyroid gland ($P < 0.05$; Table 1). Whereas, the two-way interaction between condition and season and the three-way interaction of conditions, seasons, and sex also had no significant effect on the area of the thyroid gland ($P > 0.05$; Table 1). Result of the sequential

Bonferroni post-hoc test for cross-sectional area is shown by asterisk in Fig. 5ii. Further, the follicular area (Fig. 5iii-iv) was significantly affected by the season of the study and the interaction between condition and season ($P < 0.05$; Table 1). Other factors and their interactions had no significant effect on the follicular area ($P > 0.05$; Table 1). Results of the sequential Bonferroni post-hoc test for the follicular area are shown by symbols in Fig. 5iv.

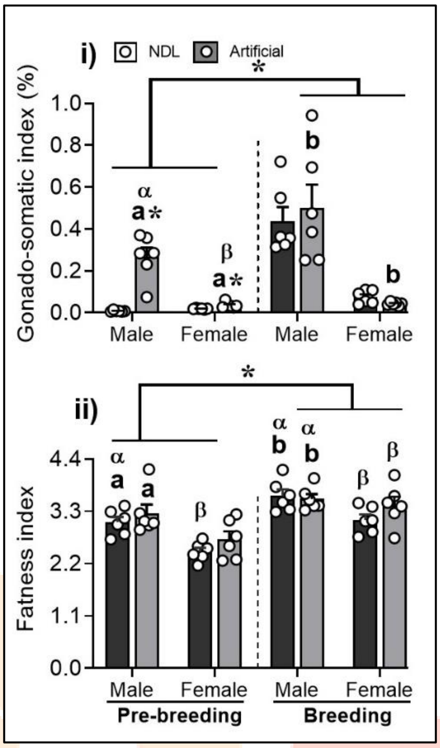

Figure 4: Graphs showing scattered dot plot and mean (± SEM) gonado-somatic index (a) and fatness index (b) in male and female redheaded buntings during pre-breeding and breeding season under natural and artificial conditions. Asterisk (*) above bar indicate significant difference between NDL and artificial condition while asterisk above line indicate overall significant difference between pre-breeding and breeding seasons. Dissimilar Greek letters over bars indicate sex-dependent differences in the same condition. While alphabets over bar indicate significant difference between pre-breeding and breeding season of the same sex.

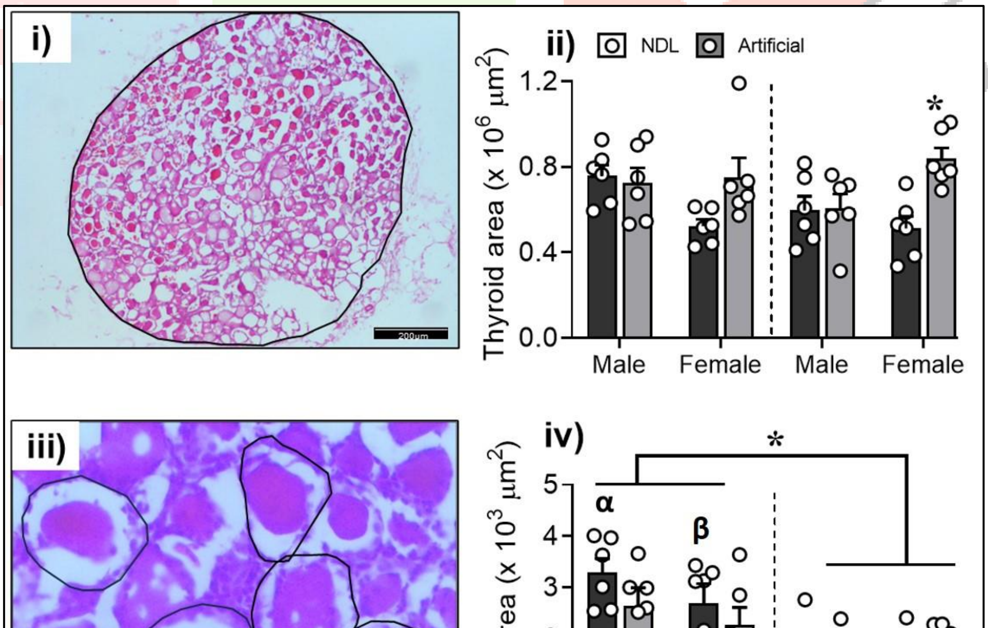

Figure 5: Photomicrographs i and iii showing marking for measurement of area of thyroid gland and thyroid follicle, respectively. Graph (ii) showing scattered dot plot and mean (± SEM) of cross-sectional area of the thyroid gland and graph (iv) is showing the scattered dot plot and mean (± SEM) of follicular area in male and female redheaded buntings during pre-breeding and breeding season under natural and artificial conditions. Asterisk (*) above bar indicate significant difference between NDL and artificial condition while asterisk above line indicate overall significant difference between pre-breeding and breeding seasons. Dissimilar

Greek letters over bars indicate sex-dependent differences in the same condition. While alphabets over bar indicate significant difference between pre-breeding and breeding season of the same sex. Scale bar: 200 μm for measurement. Image (iii) was taken at 50 μm for representation purpose.

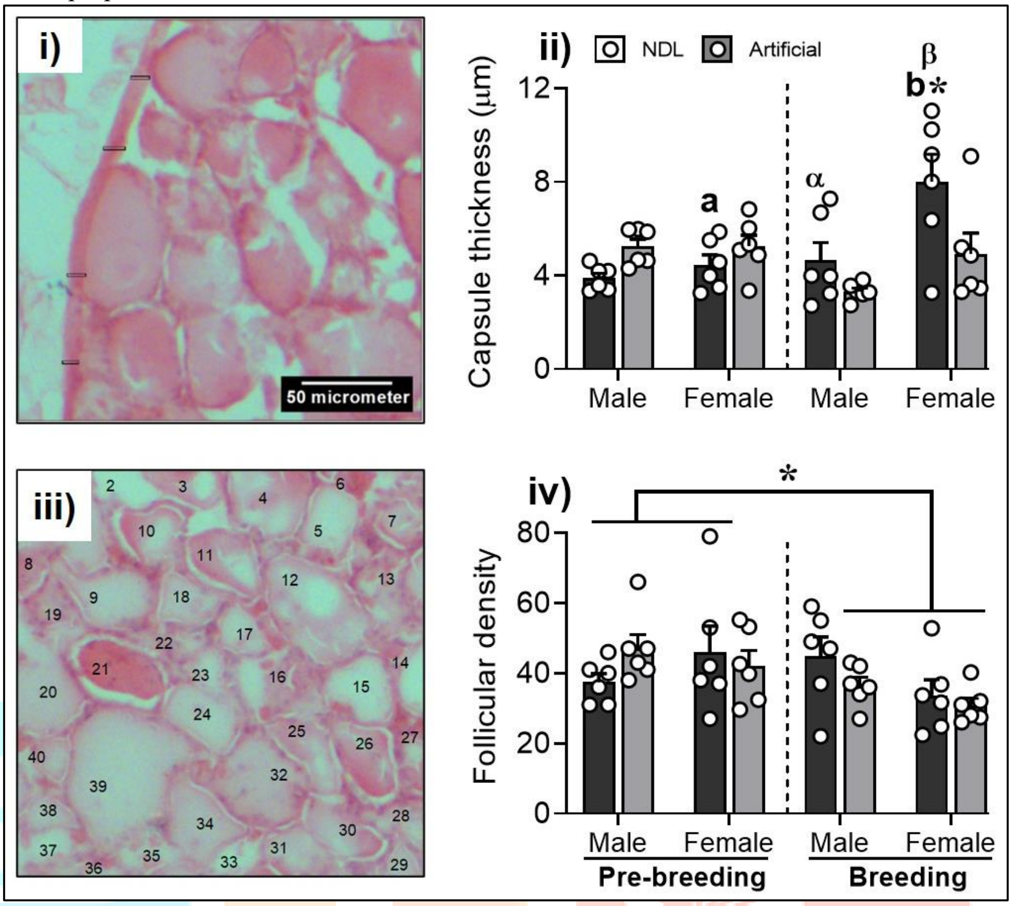

Figure 6: Cropped photomicrographs i and iii showing marking for measurement of thyroid capsule thickness and follicle density in a 221 x221 μm^2 area, respectively. Graph (ii) showing scattered dot plot and mean (± SEM) of thickness of the thyroid capsule and graph (iv) is showing the scattered dot plot and mean (± SEM) of follicular density in male and female redheaded buntings during pre-breeding and breeding season under natural and artificial conditions. Asterisk (*) above bar indicate significant difference between NDL and artificial condition while asterisk above line indicate overall significant difference between pre-breeding and breeding seasons. Dissimilar Greek letters over bars indicate sex-dependent differences in the same condition. While alphabets over bar indicate significant difference between pre-breeding and breeding season of the same sex. Scale bar: 50 μm for representation.

Table 1: Results of Generalised Linear Model (GzLM) analysis showing Wald chi-square value (χ^2) and alpha (*P*-value) of effect of factors and their interactions on parameters under consideration. Values in bold indicate the points of significant differences. Alpha was set at 0.05 for statistical significance.

	Parameters					
Factors	***Gonado-somatic index***	***Fatness index***	***Thyroid area***	***Capsule thickness***	***Follicular area***	***Follicular density***
Condition	**χ^2 = 35.522 *P*= 0.000**	χ^2 = 3.601 *P*= 0.058	χ^2 = 10.671 *P*= 0.001	χ^2 = 1.846 *P*= 0.174	χ^2 = 0.879 *P*= 0.348	χ^2 = 0.266 *P*= 0.606
Season	**χ^2 = 11.343 *P*= 0.001**	**χ^2 = 38.782 *P*= 0.000**	χ^2 = 1.544 *P*= 0.214	χ^2 = 1.555 *P*= 0.212	**χ^2 = 30.139 *P*= 0.000**	**χ^2 = 5.641 *P*= 0.018**
Sex	**χ^2 = 51.631 *P*= 0.000**	**χ^2 = 24.831 *P*= 0.000**	χ^2 = 0.153 *P*= 0.696	**χ^2 = 11.096 *P*= 0.001**	χ^2= 0.915 *P*= 0.339	χ^2= 1.238 *P*= 0.266
Condition*Season	χ^2 = 0.989 *P*= 0.320	χ^2 = 0.269 *P*= 0.604	χ^2 = 0.736 *P*= 0.391	**χ^2 = 15.577 *P*= 0.000**	**χ^2 = 4.348 *P*= 0.037**	χ^2 = 2.110 *P*= 0.146
Condition*Sex	**χ^2 = 27.831 *P*= 0.000**	χ^2 = 1.529 *P*= 0.216	**χ^2 = 12.580 *P*= 0.000**	χ^2 = 1.932 *P*= 0.165	χ^2 = 0.180 *P*= 0.671	χ^2 = 0.477 *P*= 0.490
Season*Sex	**χ^2 = 10.104 *P*= 0.001**	χ^2 = 2.073 *P*= 0.150	**χ^2 = 4.766 *P*= 0.029**	**χ^2 = 6.966 *P*= 0.008**	χ^2 = 3.080 *P*= 0.079	χ^2 = 3.175 *P*= 0.075
Condition*Season *Sex	χ^2 = 0.783 *P*= 0.376	χ^2 = 0.694 *P*= 0.405	χ^2 = 0.110 *P*= 0.740	χ^2 = 0.529 *P*= 0.467	χ^2 = 0.038 *P*= 0.846	χ^2 = 2.697 *P*= 0.101

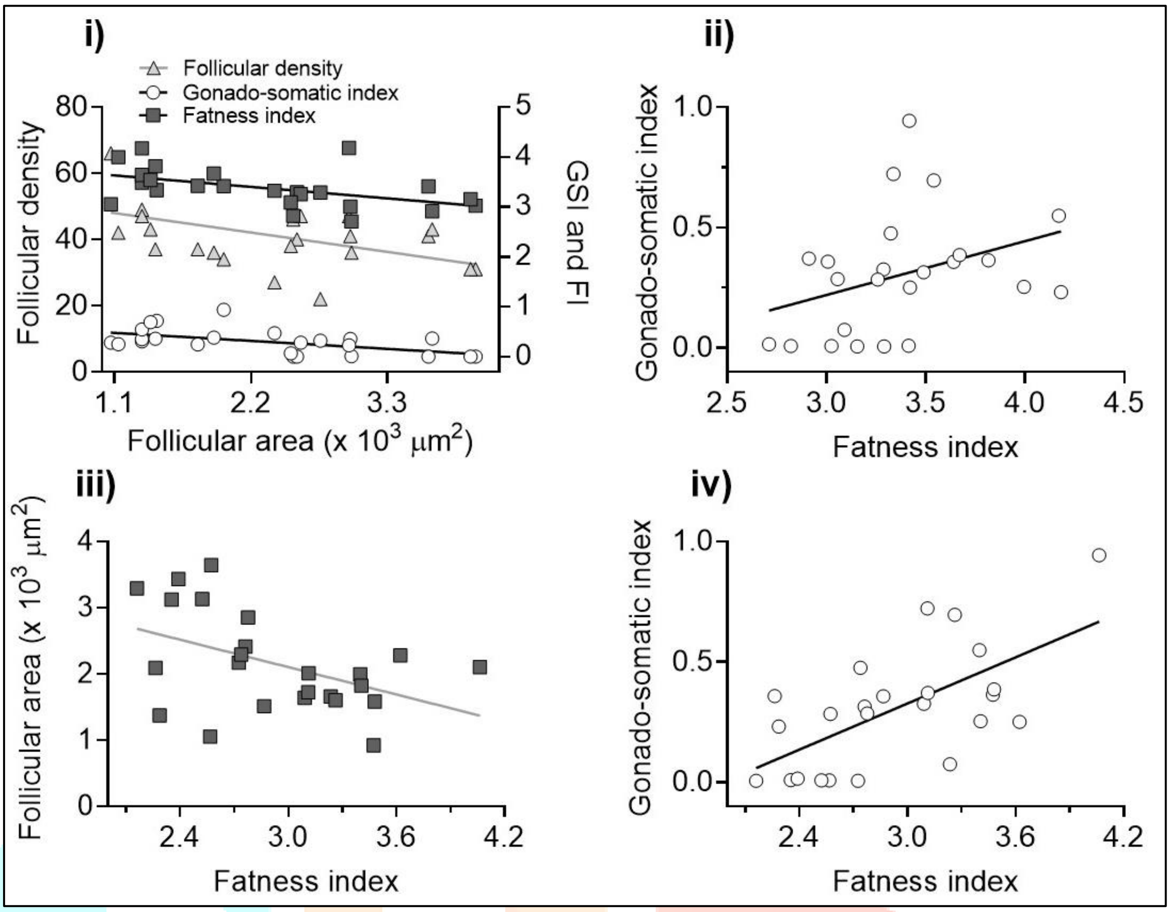

Figure 7: Graph showing Spearman correlation in male redheaded buntings between thyroid follicular area and follicular density, gonado-somatic index (GSI) and fatness index (i) and fatness index and GSI (ii) while graph (iii) is showing correlation between fatness index and follicular area and graph (iv) is showing the relationship between fatness index and GSI in female redheaded buntings during the study under natural and artificial conditions.

We also tested the thickness of the collagenous capsule (Fig. 6i-ii) surrounding the thyroid gland. The capsule thickness showed significant sex-dependent variation ($P < 0.05$; Table 1) and it was also significantly affected by the interaction between condition and season ($P < 0.05$; Table 1). Other factors and their interactions had no significant effect on the capsule thickness ($P > 0.05$; Table 1). Results of the sequential Bonferroni post-hoc test for capsule thickness are shown by symbols in Fig. 6ii. On the other hand, a comparison of the follicular density indicated that it was only affected by the season of the study ($P < 0.05$; Table 1) and other factors, and their interactions had no significant effect on the follicular density ($P > 0.05$; Table 1). Results of the sequential Bonferroni post-hoc test for follicular density is shown by symbols in Fig. 6iv.

3.3. Correlation analysis

The correlation matrix of male birds showed a negative correlation between GSI and follicular area, between fatness index and follicular area, and between follicular area and follicular density ($P < 0.05$; Fig. 7i), while a positive correlation was seen between GSI and fatness index ($P < 0.05$; Fig. 7ii); stats shown in Table 2. Moreover, in female birds, similar to male birds, a negative correlation was observed between fatness index and follicular area ($P < 0.05$; Fig. 7iii), while a positive correlation was seen between GSI and fatness index ($P < 0.05$; Fig. 7iv); stats shown in Table 3.

Table 2: Results showing correlation matrix of the study parameters analyzed by Spearman correlation for male redheaded buntings showing correlation value (r) and alpha (P-value). Values in bold indicate significant relationship between the correlated factors. Alpha was set at 0.05 for statistical significance.

	GSI	Fatness index	Follicular area	Follicular density
GSI	r = 1.000 *P* = 0.000			
Fatness index	**r = 0.599** ***P* = 0.002**	r = 1.000 *P* = 0.000		
Follicular area	**r = -0.735** ***P* = 0.000**	**r = -0.631** ***P* = 0.001**	r = 1.000 *P* = 0.000	
Follicular density	r = 0.267 *P* = 0.208	r = -0.033 *P* = 0.878	**r = -0.493** ***P* = 0.014**	r = 1.000 *P* = 0.000

Table 3: Results showing correlation matrix of the study parameters analyzed by Spearman correlation for female redheaded buntings showing correlation value (r) and alpha (P-value). Values in bold indicate significant relationship between the correlated factors. Alpha was set at 0.05 for statistical significance.

	GSI	Fatness index	Follicular area	Follicular density
GSI	r = 1.000 *P* = 0.000			
Fatness index	**r = 0.601** ***P* = 0.002**	r = 1.000 *P* = 0.000		
Follicular area	r = -0.330 *P* = 0.115	**r = -0.409** ***P* = 0.047**	r = 1.000 *P* = 0.000	
Follicular density	r = -0.223 *P* = 0.294	r = -0.345 *P* = 0.098	r = -0.337 *P* = 0.108	r = 1.000 *P* = 0.000

4.0. Discussion

Thyroid hormones (THs) play a pivotal role in regulating growth, development, and normal physiological functions, with their secretion being tightly controlled by the hypothalamic-pituitary-thyroid (HPT) axis. When comparing endocrine systems across species, THs function as permissive hormones that facilitate key transitions and plasticity in biological processes, for example, the seasonal breeding cycle (Jain & Kumar, 1995) and amphibian metamorphosis (Crespi & Denver, 2005). The thyroid gland has been extensively studied in passerine birds, including buntings, due to its critical role in regulating metabolism, reproduction, and molting (Pathak & Chandola, 1982; Pati & Pathak, 1986; Singh et al., 1992). In buntings, thyroid activity is known to exhibit seasonal variations that correlate with changes in photoperiod and reproductive status (Jain & Kumar, 1995). The present study investigated the histomorphometry of the thyroid gland in the redheaded bunting (*Emberiza bruniceps*), a small passerine migratory bird, in relation to GSI, fatness index with respect to seasons of the study, housing condition, sex of the birds, and he interaction of these factors.

Gonado-somatic index (GSI) and fatness index

The GSI, a measure of reproductive investment, was significantly influenced by the main effects of the condition of the study, seasons, and the sex of the bird, with higher GSI during the pre-breeding season relative to the breeding season. The higher GSI observed during the breeding season suggests an upregulation of reproductive activity in response to favorable environmental conditions, which is consistent with the need for optimal timing of reproduction to ensure offspring survival (Hahn et al., 1997). Morphologically, the sex-dependent variation in GSI is perhaps due to the higher weight of testes in breeding season than ovary, however, physiologically it might represent the differences in reproductive strategies between males and females, with males typically have to invest more in courtship and territorial behaviors, while females have to allocate resources to egg production and incubation (Ketterson & Nolan, 1992). This finding aligns with previous studies demonstrating that reproductive physiology in birds is closely tied to photoperiod, temperature, and resource availability (Dawson, 2008; Wingfield et al., 2016).

Similarly, the fatness index, an indicator of energy reserves, was influenced by the main effects of pre-breeding and breeding seasons and the sex of the bird, with a higher fatness index during breeding season and males showing a higher response than female birds. Migratory birds rely heavily on fat stores to fuel long-distance flights, and the observed seasonal variation in fatness index reflects the preparation for migration during the pre-breeding season and the utilization of energy reserves during the breeding season (Bairlein, 2002). The sex-dependent differences in fatness index may be attributed to LHS dependent varying energy demands between males and females during reproduction, with females often requiring higher energy reserves for egg production and incubation (Williams, 2012). An index of this kind might also be influenced by fluctuations in muscle mass and the amount of food present in the digestive tract. However, it is well-established that changes in body mass during the winter season or non-migratory season are primarily linked to variations in fat reserves (Blem, 1990; Pravosudov et al., 1999).

Both male and female showed a negative relationship between GSI and thyroid follicular area, which affirms that during reproductively active state, the thyroid follicles have little role to play. While in male as well as female, the GSI showed a positive correlation with the fatness index, which indicated that during breeding seasons, birds were equipped with ample energy reserves to meet the energetic demands of reproductive and post-reproductive events.

Thyroid gland histomorphometry

The cross-sectional area of the thyroid gland was affected by the interaction between study conditions and sex of the birds, as well as the interaction between seasons and sex of the birds. This suggests that thyroid gland activity is modulated by both surrounding conditions and reproductive factors, with sex-specific responses to the ambiance. The thyroid gland plays a critical role in regulating metabolism, thermogenesis, and molting in birds, and its morphology is known to vary in response to physiological demands (McNabb, 2007). The observed sex-dependent differences in thyroid gland size may reflect variations in metabolic rates and energy requirements between males and females during different LHSs (Chastel et al., 2003).

On the other hand, the area of thyroid follicles was influenced by the main effect of seasons; with larger active follicles during the pre-breeding season relative to the breeding season, as well as the interaction between study conditions and seasons. Thyroid follicles are the functional units of the gland, responsible for the synthesis and secretion of thyroid hormones (T3 and T4), which regulate metabolism and energy homeostasis (Decuypere et al., 2005). The observed seasonal variation in follicular area likely reflects changes in thyroid hormone production in response to shifting physiological demands, such as increased metabolic activity during the breeding season (McNabb, 2007). The interaction between study conditions and seasons suggests that environmental factors, such as temperature and photoperiod, modulate thyroid gland activity in a season-dependent manner. The findings of the present study are consistent with the earlier observations, demonstrating that thyroid functioning in redheaded buntings is influenced by seasonal and environmental factors (Sharma et al., 2018).

Similarly, follicular density was affected only by pre-breeding and breeding seasons with a greater number of follicles during the pre-breeding season than the breeding season or smaller size follicle during the pre-breeding season while larger follicles during the breeding season. This suggests that the number of follicles is primarily regulated by seasonal changes rather than surrounding environmental conditions. This finding is consistent with the role of thyroid hormones in coordinating seasonal physiological transitions, such as migration, reproduction, and molting (Dawson, 2008). The lack of an environmental effect on follicular density suggests that the thyroid gland may prioritize functional adaptation over structural changes in response to environmental stressors. A negative correlation between follicular density and follicular area indicated that as the area of cell or follicular size increased, their number decreased or less number of follicles were occupied in the given space.

Interestingly, thickness of the capsule of the thyroid gland exhibited sex-dependent variation, which may be related to differences in connective tissue composition or overall glandular activity between males and females. The thyroid capsule serves as a protective barrier and might undergo structural changes in response to hormonal fluctuation and seasons. Further research is needed to elucidate the functional significance of these sex-specific differences in thyroid capsule thickness. However, age-related progressive relation of thyroid capsule thickness has been demonstrated in Kuttanad ducks (Firdous et al., 2012) and chicken (Paul et al., 2011).

The findings reveal significant interactions between thyroid gland morphology, reproductive physiology, and housing conditions, highlighting the complex interplay between endocrine regulation, energy metabolism, and seasonal adaptations in migratory birds. The observed sex-specific and season-specific variations in thyroid gland morphology underscore the importance of considering individual and temporal factors when studying endocrine regulation in birds. The interaction between environmental conditions and thyroid gland activity has important implications for understanding the impacts of climate change on migratory birds. As global temperatures rise and weather patterns become more unpredictable, migratory birds may face challenges in synchronizing their physiological processes with environmental cues (Visser et al., 2012) and disruptions in thyroid gland function could have cascading effects on metabolism, reproduction, and survival, potentially threatening the long-term viability of migratory bird populations.

The interaction between thyroid gland size and sex-specific factors observed in this study aligns with previous research on other passerine species, which has shown that thyroid hormone levels and glandular activity can vary between males and females in response to reproductive demands (Chastel et al., 2003). Additionally, the seasonal changes in follicular area and density observed in this study are in agreement with earlier findings which have highlighted the role of thyroid hormones in mediating seasonal transitions (reviewed by Nair et al., 1994; Sharma et al., 2018). The outcomes of the interaction between the study factors and thyroid gland morphology of this migratory bird have important implications for understanding the regulation of endocrine activity in migratory bird with respect to the environmental conditions. Disruptions in thyroid gland function could have cascading effects on activity, metabolism, reproduction, and survival, potentially threatening the long-term viability of migratory bird populations.

Conclusion

In conclusion, this study provides novel insights into the histomorphometry of the thyroid gland in redheaded buntings and its relationship with reproductive physiology and ambient conditions. The findings demonstrate that histology of thyroid gland is influenced by a complex interplay of seasonal, environmental, and sex-specific factors, reflecting the adaptive strategies of migratory birds to meet the energetic demands of migration and reproduction. These results contribute to our understanding of the physiological mechanisms underlying seasonal adaptations in birds and highlight the potential impacts of surrounding factors on endocrine regulation. Future research should explore the molecular and hormonal mechanisms underlying the observed changes in thyroid gland morphology, as well as the long-term effects of environmental stressors on thyroid function and reproductive success. Additionally, comparative studies across different migratory and non-migratory species could provide further insights into the evolutionary significance of thyroid gland adaptations in birds. By advancing our understanding of the endocrine regulation of migration and reproduction, this research has important implications for the conservation and management of migratory bird populations in a changing world.

5.0. References

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported by the authors.

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Ethical approval

Institutional Animal Ethics Committee, University of Lucknow, approved the study via protocol no.: # LU/ZOOL/IAEC/01/21/02 for carrying out this study.

Data availability

Datasets will be made available upon reasonable request from the corresponding author Prof. Shalie Malik.