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Histological evaluation of the development of the thymus and spleen in the light ecotype Nigerian indigenous chicken (*Gallus gallus domesticus*)

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Abstract

The Nigerian indigenous chicken (NIC) is a hardy breed that has been widely reported to be very resistant to diseases. Though the thymus and spleen are functionally very significant in the chicken, their morphological development in the NIC has not been reported. The present study histologically evaluated the morphological development of the thymus and spleen of light ecotype Nigerian indigenous chicken. Fifteen fertile eggs of the light ecotype NIC (Group A) and 15 light ecotype NICs (Group B) were used for the study. The eggs were sampled on embryonic days (ED) 14 and 19, and post-hatch day (PD) 1, while the chickens were chronologically sampled according to the following groupings: B1 (3 – 6 months of age; 210 – 250 g; n = 5), B2 (6 – 9 months of age; 400 – 600 g weight; n = 5), and B3 (12 – 18 months of age; 900 – 1200; n = 5). Samples of the thymus and spleen were collected at the specified time points, processed and stained for histological evaluation following standard procedures. Results of the histological evaluation showed that the thymus at ED 14 contained loose connective tissue areas with numerous islands of nascent thymic tissues. At ED 19, the thymic lobules showed large cortical areas with small medulla, whereas, the thymus at PD 1 had distinctly demarcated parenchymal areas. The thymus of 3 – 18 months old birds showed extensive medullary area that contained diffusely distributed Hassall's corpuscles and necrotic foci. The spleen, at ED 14, contained red pulp areas with cords of Billroth and occasional shallow sinusoids. Wide splenic sinusoids containing numerous red blood cells occurred at ED 19. However, at hatch, the splenic parenchyma had clearly demarcated red and white pulps with predominance of white pulp areas in the spleen of older birds. It was concluded that the thymus of the light ecotype Nigerian indigenous chicken is structurally mature in the late embryonic and early post-natal life, with regressive changes occurring around 3 months of age, but the spleen may predominantly perform hematopoietic function during embryonic life, with the predominant role of immune function ensuing after hatch.

Keywords: Embryonic development; Morphology; Histology; Nigerian indigenous chicken; Spleen; Thymus.

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Introduction

The Nigerian indigenous chicken (*Gallus gallus domesticus*), a Galliformes, in the Phasianidae family, is a widely bred breed of chicken in Nigeria (Sanda *et al*., 2012). The scavenging, gregarious social attributes of the bird, as well as the climate change-induced harsh environmental conditions expose them to dangerous pathogens as they scavenge (Nwogwugwu *et al*., 2018). However, The Nigerian indigenous chicken, among other local breeds of chicken, is hardy in nature and has been widely reported to be resistant to diseases (Xiang *et al*., 2014; Mpenda *et al*., 2019). The genetic diversity of the local chicken as well as the role of their lymphoid organs have been suggested as the basis of such disease resistance (Olah *et al*., 2014; Okumu *et al*., 2017; Samaraweera *et al*., 2021). Studies on the genetic diversity of the breed have received increasing attention over the years, but little has been done to evaluate the morphological development of their lymphoid organs. It is believed that investigation of the embryonic and post-embryonic structural features of the primary and secondary lymphoid organs of the breed may provide strategic clues for their effective management.

The thymus, bursa of Fabricius and bone marrow are considered as the primary lymphoid organs while the spleen and all mucosa-associated lymphatic tissues are the peripheral or secondary lymphoid tissues. These lympho-myeloid tissues develop from epithelial or mesenchymal enlages, and are thereafter colonized by blood-borne haematopoietic cells (Moticka, 1975; Olah *et al*., 2014). In chicken, the thymic enlage has been reported to form at about 2 or 3 days of incubation, becoming colonized by haematopoietic stem cells at day 10 of incubation (Moticka, 1975). These cells differentiate to form the immunologically competent T cells that will home to secondary lymphoid organs. Recent reports argue that the development of thymic T cells in the young is directly related to changes in the gut microbiota (Akinyemi *et al*., 2020; Ennamorati *et al*., 2020; Cheng *et al*., 2021). Thus, the changing alpha-diversity of the gut microbiota will impede or accelerate the maturation of humoral immunity (Cheng *et al*., 2021). In the Nigeria indigenous chicken, their scavenging habit may enhance their gut alpha-diversity which will in turn influence the timing of colonization, maturation and regression of the thymus.

The splenic enlage has been reported to develop from the dorsal mesogastrium and are colonized by hematopoietic cells at about day 6.5 of incubation (Olah *et al*., 2014, Zhang *et al*., 2015). Further embryonic and post-embryonic development of the spleen like most secondary lymphoid organs is dependent on antigen exposure which is largely regulated by changing environmental factors (John, 1994; Olah *et al*., 2014). Intra-specific variation in size of avian spleen which has been linked to changing seasons has been reported to influence the splenic morphology and activity (John, 1994). The absence of lymph nodes in avians positions the spleen as a principal organ of immunity. Although avian spleen is little-studied when compared with the mammalian spleen, there is increasing focus on the functional significance of the organ (Nagy *et al*., 2005; Kita, 2014), and there is little or no information on the variability of spleen morphology of varied breeds of local chicken. It is thought that the evaluation of the splenic development of this roaming Nigerian chicken breed may inform of important time-points during their development and growth that can be harnessed for effective management and disease control. The present study evaluated the morphological development of the thymus and spleen in light ecotype Nigerian indigenous chicken.

Materials and Methods

Materials: Fifteen fertile eggs (Group A) of the light ecotype Nigerian indigenous chicken (NIC) and fifteen post-hatch chickens (Group B) were used for the study. The fertile eggs (Group A) were obtained from households in Nsukka Local Government Area, Enugu State, Nigeria, while the post-hatch birds (Group B) were purchased from local markets in Nsukka Local Government Area, Enugu State, Nigeria. The fertile eggs were incubated in the Department of Veterinary Anatomy, University of Nigeria, Nsukka, Enugu State, Nigeria, and spleen and thymus samples were collected on embryonic days (EDs) 14 and 19 and post-hatch day 1. The post-hatch light ecotype NICs (Group B) used for the study were further grouped into three: Group B1, aged between 3 – 6 months, weighing 210 – 250 g (n = 5); Group B2, aged between 6 – 9 months, weighing 400 – 600 g (n =5); and Group B3, aged between 12 – 18 months, weighing 900 – 1200 g (n = 5).

The embryos or birds were humanely sacrificed using 13 mg/kg ketamine hydrochloride injection, and samples of the spleen as well as thymus were collected and processed for histological evaluation. The guidelines regarding the care and use of animals were strictly adhered to all through the study. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Faculty of Veterinary Medicine, University of Nigeria, Nsukka (Approval Reference Number: FVM-UNN-IACUC-2019-077).

Histological procedures: Samples of the thymus and spleen that were collected from the embryos or chicks were fixed by immersion in 10% neutral-buffered formalin. The fixed tissues were dehydrated in increasing concentrations of ethanol and thereafter cleared in three changes of xylene (at 60 mins intervals). After clearing, the tissues were embedded in paraffin wax and mounted in wooden blocks for sectioning with a rotary microtome. The 5 – 6 µm-thick sections obtained were stained with haematoxylin and eosin (H & E), and reticulin stain for light microscopy. The H & E staining procedures was performed according to the methods of Sheehan and Hrapchak (1980), while the reticulin staining procedures were carried out as described by Lefkowitch (2006). Photomicrographs were captured using Moticam images plus 2.0 camera (Motic group Ltd) attached to the motic binocular light microscope.

Results

Thymus*:* At embryonic day 14, the thymus showed loose connective tissue areas with numerous islands of nascent thymic tissues (Figure 1). The cortical and medullary areas of the thymic lobules were poorly defined with adjacent stromal areas containing network of blood vessels (Figure 1). At embryonic day 19, the thymic lobules showed large cortical areas of thymocytes and small medullary areas (Figure 2). At post-hatch day 1, the distinctly demarcated thymic lobules contained large cortical areas and narrow lightly-stained medulla (Figure 2).

The thymus of the 3 to 9 months old chickens had extensive medullary areas that contained diffusely distributed Hassall's corpuscles and necrotic foci (Figure 3). As the thymus aged (at 12 – 18 months), each thymic lobule showed extensive area of thymic medulla that was surrounded by thin marginal cortex, and the thymic parenchyma contained numerous terminally differentiated lymphocytes, Hassall's corpuscles (HC), apoptotic cells and blood capillaries (Figure 3).

Spleen: At embryonic day 14, the cords of Billroth predominated in the red pulp areas with occasional shallow areas of splenic sinusoids that contained isolated or aggregates of red blood cells (Figure 4). At embryonic day 19, the wide splenic sinusoids

of the red pulp were distinctly demarcated and contained numerous red blood cells.

At post-hatch day 1, the splenic parenchyma showed well demarcated white pulp and red pulp areas, while the spleen of the light ecotype Nigerian indigenous chicken between the ages of 3 to 9 months showed extensive white pulp areas that were characterized by the presence of areas of dense lymphocytic infiltration with lymphoid nodules and dense accumulation of lymphocytes arranged around the central arteries (PALS – peri-arteriolar lymphoid sheath) (Figure 4).

Reticulin histochemistry of the spleen revealed the presence of sparse network of reticular fibres in the capsular areas and within the splenic tissues at embryonic day 19. At hatch, the splenic capsules and trabeculae were composed of dense network of reticular fibres while the splenic tissues containing sparsely distributed network of reticular fibres (Figure 5).

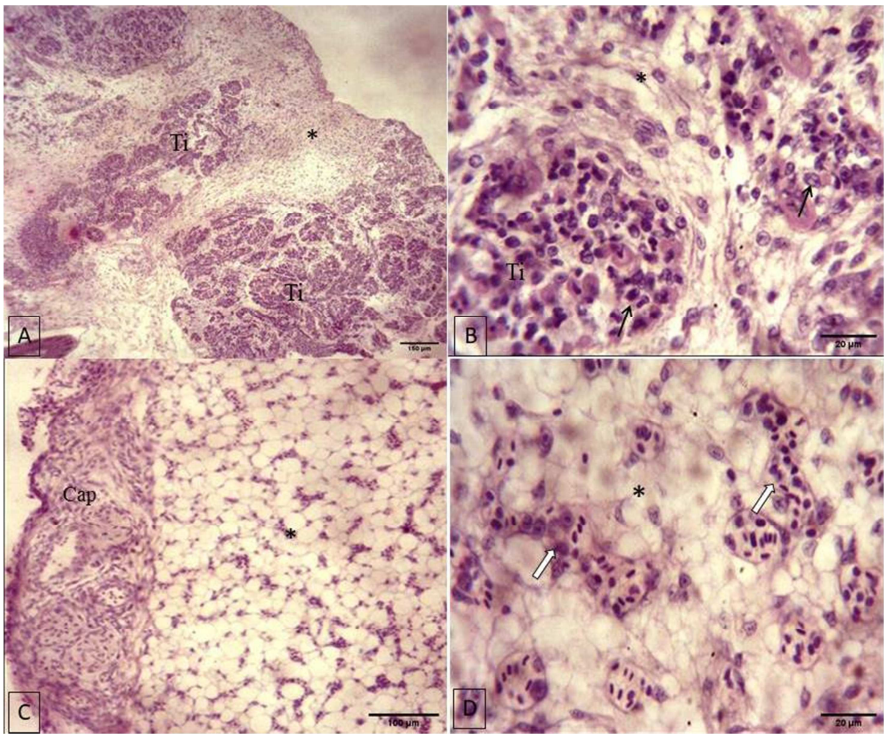

Figure 1: The thymus of Nigerian indigenous chicken at embryonic day 14 showing: **A –** Islands of nascent thymic tissues (Ti), and area of loose connective tissue (asterisk), H & E × 100; **B –** Islands of thymic tissues (Ti), thymocytes (black arrows) and loose connective tissue area (asterisk), H & E stain, × 400; **C –** Thymic capsule (Cap), and loose connective tissue area (asterisk), H & E stain, × 100; and **D –** Area of loose connective tissue (asterisk) and blood vessels (white arrows). H & E stain × 400.

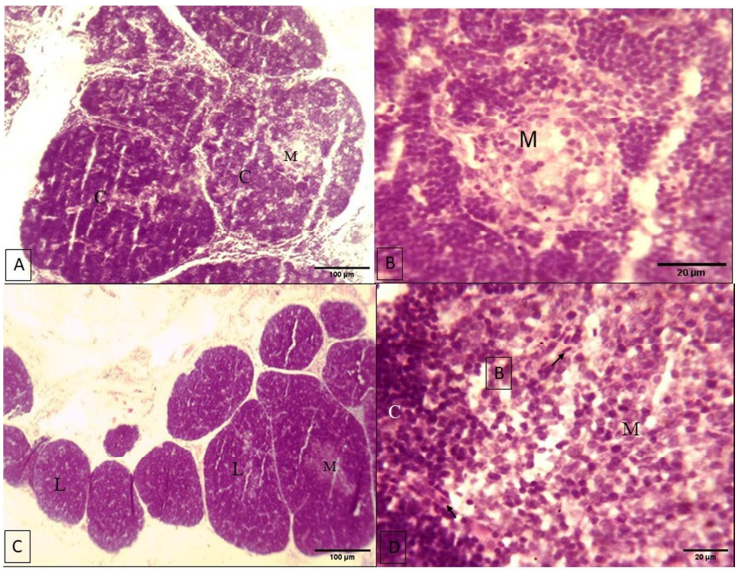

Figure 2: The thymus of Nigerian indigenous chicken: **A –** At embryonic day 19 showing thymic lobules with cortical (C) and medullary (M) areas, H & E × 100; **B – A**t embryonic day 19 showing small medullary area (M), H & E stain, × 400; **C** – At post-hatch day 1 showing distinctly demarcated thymic lobules (L) containing mostly cortex and small medulla (M), H & E stain, × 100; and **D –** At post-hatch day 1 showing thymic cortex (C) and medulla (M), H & E stain, × 400.

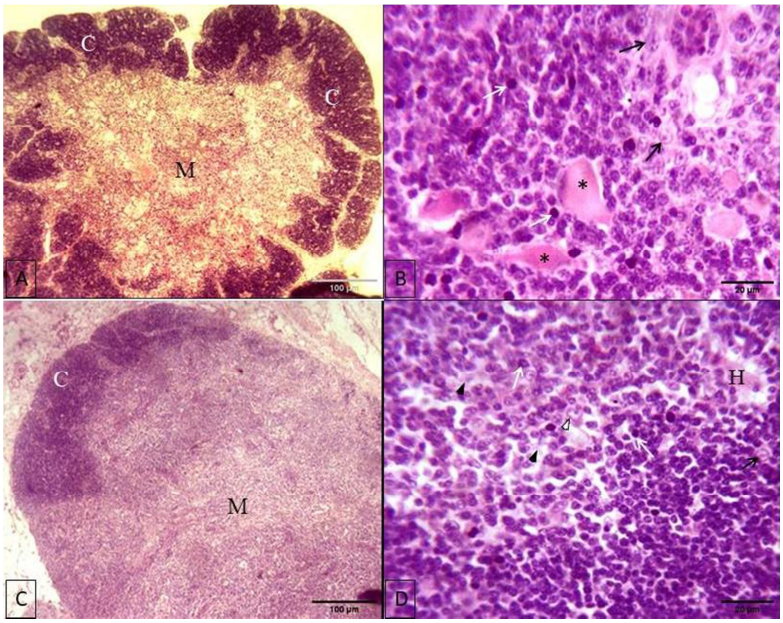

Figure 3: The thymus of Nigerian indigenous chicken: **A –** Thymus of 3 – 9 months old chickens showing thymic cortex (C) and medulla (M), H & E × 100; **B** – Thymus of 3 – 9 months old chickens showing thymic medulla with Hassall's corpuscles (asterisks), apoptotic thymocytes (white arrows) and necrotizing epithelial reticular cells (black arrows), H & E stain, × 400; **C –** Thymus of 12 – 18 months old chickens showing thin marginal cortex (C) and extensive medulla (M), H & E stain, × 100; and **D** – Thymus of 12 – 18 months old chickens showing thymic medulla with epithelial reticular cell (black arrow), necrotizing epithelial reticular cell (white arrow head), apoptotic thymocytes (white arrows), Hassall's corpuscle (H) and necrotic areas (black arrow heads), H & E stain × 400.

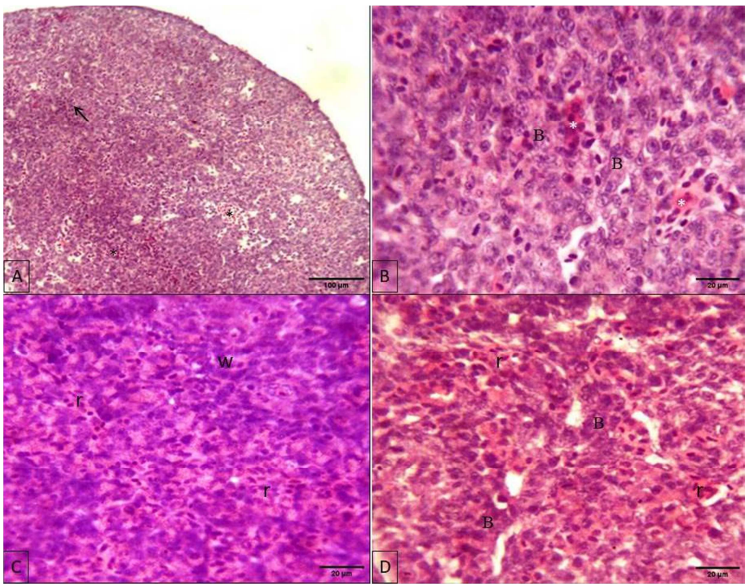

Figure 4: The spleen of Nigerian indigenous chicken: **A –** At embryonic day 14 showing isolated white (arrow) and red (asterisks) pulp areas, H & E × 100; **B -** At embryonic day 14 showing the dominance of the cords of Billroth (B) in the red pulp areas. Note the isolated splenic sinusoids (asterisks), H & E stain, × 400; **C –** At post-hatch day 1 showing white (w) and red (r) pulp areas, H & E stain × 400; and **D** - At post-hatch day 1 showing cords of Billroth (B), splenic sinusoids with red blood cells (r), H & E stain × 400.

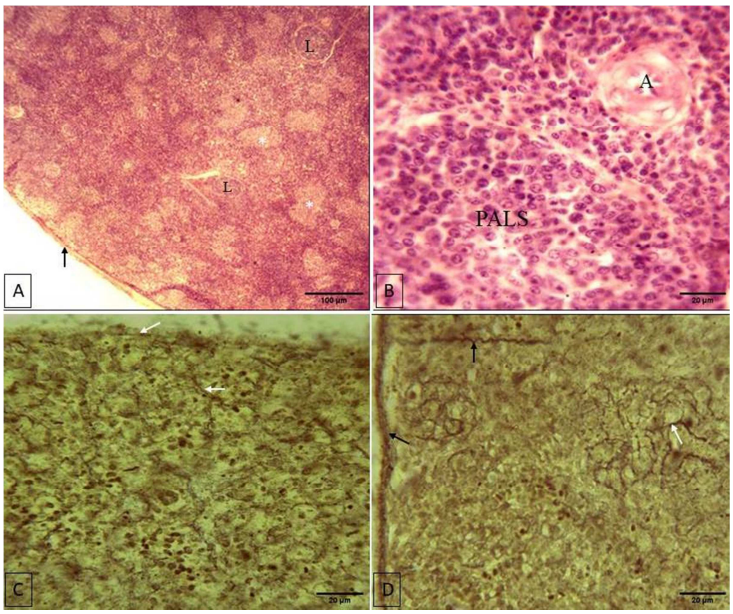

Figure 5: The spleen of Nigerian indigenous chicken: **A** – Spleen of 3 – 18 months old showing splenic capsule (arrow), white pulp areas (asterisks) and lymphoid nodules (L), H & E stain × 40; **B** – Spleen of 3 – 18 months old showing an arteriole (A) and peri-arteriolar lymphoid sheath (PALS), H & E stain × 400; **C** – Spleen at embryonic day 19 showing sparse distribution of reticular fibres in the capsule and parenchyma (arrows), Reticulin stain × 400; and **D** – Spleen at post-hatch day 1 showing dense network of reticular fibres in the capsule and trabeculae (black arrows) and spare reticular fibres within the spleen (white arrow), Reticulin stain × 400.

Discussions

The thymus plays key roles in the development of immunocompetence; thus, a dysfunctional development of the organ will result in immunodeficiencies. In this study, the presence of islands of nascent thymic tissues at ED 14 suggests focal distribution of the infiltrating T-cell progenitors. Previous reports showed that T-cell progenitors colonize the epithelial enlage of the thymus in 3 successive waves viz: 1st, 2nd and 3rd waves (Mehr *et al*., 1994; Ashby and Hogquist, 2024). The ED 14 in the current study coincides with the 2nd wave of cellular infiltration earlier reported in chick and quail embryos, where T-cells originate from the bone marrow (Mehr *et al*., 1994). The absence of distinct thymic zones at ED 14 in this study further indicates T-cell colonization and maturation phase of thymus. Although thymic lobules were observed at ED 19, the lobules were more distinct at PD 1. The presence of distinct large cortical and thin medullary areas between ED 19 and post-hatch day 1 in this study suggests structural maturation of the organ. It has been reported that the formation of thymic cortex coincides with the emigration of T cells from the thymus to secondary lymphoid organs (Weinreich and Hogquist, 2008; Fellah *et al*., 2014). The structural maturation of the thymus at late embryonic life and early post-hatch periods as recorded in this study is in agreement with earlier reports of fully functional thymus at these periods of lymphoid tissue development (Glick, 1978).

In the early stages of development of the thymus in the light ecotype Nigerian indigenous chicken, it appears that the thymic cortex predominates. It is believed that thymocytes in the thymic cortex are less mature than thymocytes in the medulla, supporting the assertion of thymocytes migration from the cortex to the medulla during T cell development (Nitta *et al*., 2011). Within the cortical microenvironment, re-arrangement of T-cell receptor α and β loci, and positive and negative selection of newly formed $CD4^+CD8^+$ thymocytes, occur, whereas, majority of thymocytes in the medulla are either $CD4^+CD8^-$ or $CD4^-CD8^+$ (Gameiro *et al*., 2010; Love and Bhandoola, 2011). As the birds aged, the cortical regions narrowed to the periphery of each thymic lobule, leaving extensive lighter-stained medullary areas in H & E-stained preparations. This was evident in this study as the thymus of 3 to 6 month-old as well as 6 to 9 months old Nigerian indigenous chicken showed extensive medullary areas that contained numerous plasma cells, diffuse Hassall's corpuscles and areas of lymphocytic necrosis. The decreased cortical region from the 3-months old chickens mirrors decreased expansion of thymocytes population, positively selected for TCR receptors that will recognize self-MHC molecules (Takada *et al*., 2014; Shichkin and Antica, 2022).

The preponderance of Hassall's corpuscles and necrotic foci in the thymic medulla of 3 to 9 months old chickens suggests involutive changes that are associated with senescence. In humans, thymic involution is reported to occur much earlier before puberty, while in birds, the timing of thymic involution varies greatly among species (Ciriaco *et al*., 2003; Shanley *et al*., 2009; Ayman *et al*., 2020). Thymic regression could be caused by several factors including age, environmental stress, nutritional and hormonal factors (Ciriaco *et al*., 2003; Glick, 1957). The observed regressive changes in the thymus of 3 to 6 months old chickens as well as 6 to 9 months chickens in the present study may be related to age and environmental factors. Wang *et al* (2019) reported that cytokines and chemokines production function of the HC-medullary thymic epithelial cells triggered the recruitment of thymic neutrophils and plasmacytoid dendritic cells (cells that secrete IFNa required for single positive T cell maturation) in the thymic medulla of mice. In the current study, the presence of thymic

neutrophils and the increasing capillary network in the thymic medulla of 3 – 9 months old Nigerian indigenous chicken may further indicate that HCs modulate the thymic microenvironment to enhance single positive thymocyte maturation in aging thymus.

The spleen in avian and mammalian species is an important hematopoietic and immune organ (Masteller and Thompson, 1994; Lewis *et al*., 2019). In this study, red pulp areas of the spleen predominated at ED 14 and 19, suggesting that the spleen in the late foetal life of the bird performs haematopoietic function. This function was previously considered to last until the late foetal period in most species (Diemert and Tutschek, 2018). The splenic red pulp, apart from the extramedullary hematopoiesis, also store RBC as well as monocytes and platelets (Bronte and Pittet, 2013; Lewis *et al*., 2019). The shallow areas of splenic sinusoids observed at ED 14 may indicate limited storage of red blood cells. The concentration of red blood cells in the splenic sinusoids increased with age in this study, meaning that the RBC storage role of the spleen is enhanced in early post-natal life. However, at post-hatch day 1, there was enhanced development of white pulp areas alongside red pulp areas. This could be a response to sudden exposure of the birds to environmental antigens. In the spleen of 3 – 6 months old birds, the morphological features of the white pulp areas were more enhanced in the present study than the red pulp areas, suggesting that the spleen of this avian species may be more adapted as an immune defense organ.

The present study showed that the reticular fibres were sparsely distributed in the chicken spleen at ED 19, such that the capsule was not obvious. The sparse distribution of reticular fibres most-likely coincides with the haematopoietic period, when the spleen structure is considered immature (Biro *et al*., 2011). It has been reported that the reticular fibre network in the spleen continues to increase with age until post-hatch day 14 in quail and duck (Xu *et al*., 2020). The scope of this study did not reveal the distribution of reticular fibres in the later ages of the studied species. However, at post-hatch day 1, dense network of reticular fibres revealed clearly delineated capsular and trabeculae areas. The reticular fibre network increased at hatch more than at ED 19, most probably preparing the spleen for immune functions. Reticular fibres and cells within lymphoid organs provide supportive network that provides a microenvironment for the trafficking of T and B lymphocytes (Pellas and Weiss, 1990; Biro *et al*., 2011; Bancroft and Layton, 2019; Xu *et al*., 2020).

Conclusion: Results of this study revealed that the thymus of light ecotype Nigerian indigenous chicken is structurally and functionally mature in the late embryonic and early post-hatch life, with regressive changes occurring around 3 months of age. The spleen at embryonic life may predominantly perform hematopoietic function. At hatch, the reticular stromal networks are fully established to support immunological functions which appear to be the predominant function of the spleen of the light ecotype Nigerian indigenous chicken after hatch.

Conflict of Interest

The authors declare that there is no conflict of interest.

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