

Histomorphological Structure of the *Bursa cloacalis* (Bursa of Fabricius) in Young *Leiothrix lutea*: A Comparative Study Using Light Microscopy and Transmission Electron Microscopy

Estructura Histomorfológica de la *Bursa cloacalis* (Bursa de Fabricius) en Jóvenes *Leiothrix lutea*: Un Estudio Comparativo Utilizando Microscopía Óptica y Microscopía Electrónica de Transmisión

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SUMMARY: This study aimed to investigate the microstructure and ultrastructure of the *Bursa cloacalis* (Bursa of Fabricius) (BC) in young *Leiothrix lutea* at various days of age (a few days after hatching) using light microscopy and transmission electron microscopy. The bird BC was sampled at 1, 5, 7, and 9 days of age. Immediately after dissection, the structure and integrity of the BC (not degenerative) were retained and the specific temporal features could be visualized precisely. After hematoxylin-eosin staining and uranyl acetate/lead citrate staining, the microstructure and ultrastructure of the BC, respectively, could be observed clearly. The microscopic observations revealed the following: in addition to change in the size of BC or lymphoid follicles, many cavities were found in the BC; the distribution of the lymphoid follicles in *Leiothrix lutea* was different from that in other birds; and the segregating line between the bursal cortex and medulla became increasingly clear as the age increased. In conclusion, the structural data obtained in this study provides a better understanding of the specific immunological function of the BC in *Leiothrix lutea*.

KEY WORDS: *Leiothrix lutea*; *Bursa cloacalis*; Bursa of Fabricius; Microstructure; Ultrastructure; Lymphoid follicles

INTRODUCTION

The *Bursa cloacalis* (Bursa of Fabricius) (BC) is a central immune organ placed in the cloacae, and is a peculiar organ unique to avian species. Interestingly, the BC can act as both the primary and secondary lymphoid organ. Functionally, it is responsible for the development and differentiation of B lymphocytes (Abbate *et al.*, 2007), and provides a milieu for the development of both myeloid and lymphoid cells (Valinsky *et al.*, 1981). Thus, the BC is related to normal humoral immune function in birds. In addition, the BC has been postulated to play a role in the immune-endocrine axis by producing a putative anti-steroidogenic factor (Garcia-Espinosa *et al.*, 2008). Therefore, the BC may be a good model organ for studies on the effects of certain factors on B-cell function.

The BC functions are closely related to its structure. Different birds have different structures of the BC. For instance, the ostrich BC is not a cloacal appendix, but a wall

organ of the caudal cloacal chamber (proctodeum). Histologically, the positions of the cortex and medulla in ostriches are different from that in other birds (such as chicken and duck), with the medulla and cortex being the outer and inner organs respectively. Furthermore, the types of mucosal epithelium in the lymphoid follicles are varied across different birds; some have stratified columnar epithelium, while others have pseudostratified columnar epithelium, single-layered columnar epithelium, or simple cuboidal epithelium. Ostrich and chicken have been reported to show some differences in the BC anatomy and histology; the unique histology of ostrich tissues illustrates a transition of the digestive tract into the BC. The normal structure of the BC has a close relationship with its function, and hence, the unique structure determines the unique functions of the BC in different birds. Thus, if the unique structure of the BC in some birds is related to disease resistance or other functions, then this requires further study.

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As a homeothermic animal, the establishment of a thermostatic mechanism in birds is consistent with the development of various organs, and it gradually improves, especially in the pullus. When the pulli of *Leiothrix lutea* are about 9 days old, their body temperature gradually obtains relative stability (Ma *et al.*, 2010; Hu *et al.*, 2010). Before this time period, pulli are vulnerable to external pathogens. The BC develops faster than the thymus and other immune organs (Zhang *et al.*, 2017) and plays an irreplaceable role in the nestling stage. With the development of the BC, the lymphoid follicles are formed, and hence, external antigens can be captured and eliminated by the BC. This process guarantees the normal physiological activities of *Leiothrix lutea* during the breeding period. Therefore, the period extending from the birth to 9 days of age is critical for the adaptation and survival of *Leiothrix lutea* (Zhang *et al.*, 2016). The BC can develop rapidly during this period and gradually exert specific immune responses, which help improve the environmental adaptability of nestlings to increase their survival rate.

Many birds live in the wild, and they are continuously exposed to pathogens, such as viruses, bacteria, or macroparasites. An individual's immune capacity is closely linked to its fitness (Saino *et al.*, 2004; Cichon & Dubiec, 2005). Accordingly, as opposed to domestic birds, wild birds need to have a strong immune capacity for defense against unwanted biological invasion, in order to meet their ecological adaptation and flexibility, or survival needs. Thus, we speculated that the different structures of the BC may be related to bird survival in the wild.

Leiothrix lutea (red-billed leiothrix) is a kind of mini type wild bird belonging to the class Aves, order Passeriformes, family Timaliidae, and genus *Leiothrix* (IUCN, 2009), and has colorful feathers approximately fifteen centimeters long (Rodrigues *et al.*, 2006). Popular as the symbol of "fidelity to love," the red-billed leiothrix, who is a member of the family Leiothrichidae and native to the Indian Subcontinent, has been classified as a wild bird having significant economic and academic research values in China. Its economic value is attributed to its beautiful appearance and pleasant-sounding chirps, which makes red-billed leiothrix popular as a common cage bird. Alternatively, it has been introduced in many countries worldwide to perform certain duties for maintaining the local biodiversity. Some studies on *Leiothrix lutea* have focused on its living environment, habitat, breeding, and feeding habits (Liu & Long, 1989; Ma *et al.*, 2010). In addition, some other researchers have studied the digestive system, chirping, and the anatomy of the skeletal system of *Leiothrix lutea* (Liao *et al.*, 2006; Qi *et al.*, 2010; Wang *et al.*, 2011).

Many investigations have been conducted on the structure and ultrastructure of the pigeon and chicken BC. However, the histomorphological structure of the BC of *Leiothrix lutea* in the early days post hatching has not been reported to date. Investigating the histomorphological structure of the BC is important to reveal its key roles in immune function. Therefore, in this study, the microstructure and ultrastructure of the *Leiothrix lutea* BC in the period extending from day 1 to 9 post birth was studied by histology and ultrastructural analysis, respectively. The results may provide insights into the histomorphological (microstructure and ultrastructure) structure and the dynamic changes in the BC few days after hatching, thereby laying the foundation for future research on the immune functioning of *Leiothrix lutea* or other birds.

MATERIAL AND METHOD

Experimental animals. Sixteen *Leiothrix lutea* birds of different ages (1, 5, 7, and 9 days after hatching; 4 birds in each age group) were obtained from an artificial training and reproduction base at the Key Laboratory of Southwest China Wildlife Resources Conservation-Ministry of Education, Chinese West Normal University (Nanchong, China). The birds were worm-fed.

General observation. The location, shape, size, and the mucosal folds of the BC were observed at different ages.

Sample collection. The birds were executed by jugular vein bloodletting. The BC of each bird was dissected immediately, and one half of the tissue was fixed in 4 % paraformaldehyde solution for at least more than 24 h for microstructure observation. The other half was fixed in 2.5 % glutaraldehyde and post-fixed in 2 % veronal acetate-buffered OsO₄ for ultrastructural observation.

Microstructure observation by light microscopy. In order to investigate the microstructure of the BC, the tissue samples were subjected to hematoxylin-eosin (HE) staining and visualized under a light microscope (Olympus, Japan) using a color video camera (Nikon 3 CCD) and an analytical software (version 4.6). After rinsing the tissue samples with water, they were dehydrated using a graded series of absolute ethanol (50 %, 70 %, 80 %, 90 %, and 100 %), clarified with benzene twice, and saturated with and embedded in paraffin. Sections of 5 mm thickness (10 slices of each sample) were stained with HE and prepared for observation under a light microscope. Four stained tissue slides from each age group (1, 5, 7, and 9 days; 16 slides in total) were observed with a total magnification of 100×, 200×, and 400×. All the structural characteristics of

the BC, especially the distribution of lymphocyte follicles, and other special features, such as the segregation line between the bursal cortex and medulla, were analyzed.

Ultrastructure observation by transmission electron microscopy (TEM). TEM was used to study the ultrastructural development of the BC. The BC tissues from birds belonging to the four age groups were dissected and fixed in 2.5 % glutaraldehyde and post-fixed in 2 % veronal acetate-buffered OsO₄. After dehydration using an alcohol gradient, the BC tissues were embedded in araldite. The tissue blocks were sectioned with a microtome using a glass blade into 65–75 nm thick fragments, which were first placed on uncoated copper grids and then stained with uranyl acetate, followed by post-staining with 0.2 % lead citrate and examination under a transmission electron microscope (H-600). With TEM, cell apoptosis and secretion, as well as other processes during the development of the BC, such as cell division and the related ultrastructures, were observed.

RESULTS

Gross anatomy. The BC is dorsally located on the proctodeal part of the cloaca. It is characterized by a single oval sac with a collum or neck cloaca, enclosing a luminal or cryptal system, which forms a slit-like opening through the collum into the cloaca.

The general morphology of the BC.
Microstructure of the BC: The shape of the BC displayed an oval saccular form. Its size, however, increases from day 1 to day 9, as shown in Figure 1. Interestingly, the distribution of the lymphoid follicles was even throughout the BC, with many lymphoid follicles constituting most of the area of the BC, which was contrasting to that observed in other birds.

Changes in the size of lymphoid follicles: The lymphoid follicles, could clearly be observed histologically from days

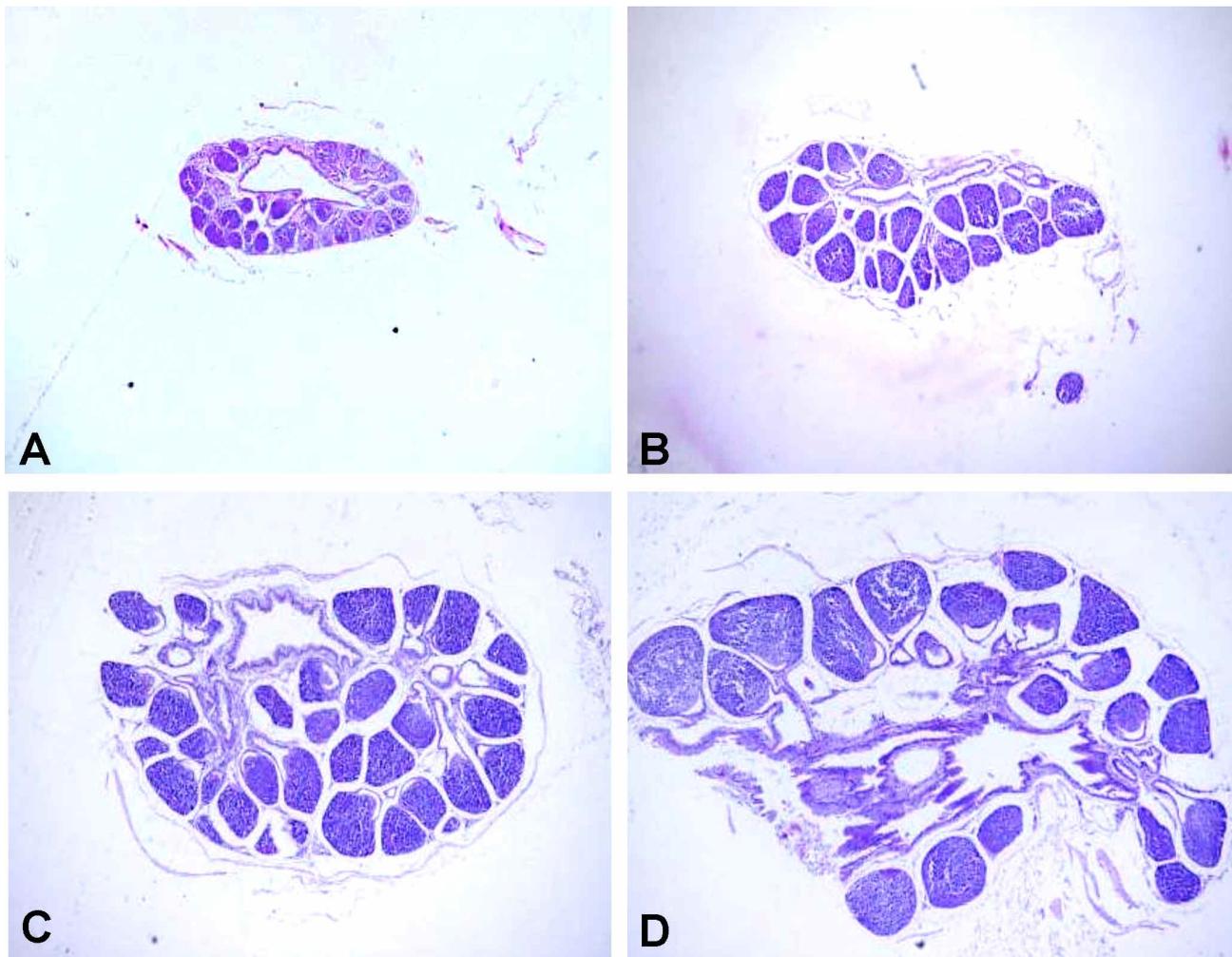


Fig. 1. A. The BC of a 1-day-old bird. B. The BC of a 5-day-old bird. C. The BC of a 7-day-old bird. D. The BC of a 9-day-old bird. Tissues were stained with hematoxylin-eosin (HE) and observed under an optical microscope.

1 to 9 (Fig. 1). The size of the lymphoid follicles, which represented the functional units of the BC, increased from days 1 to 9 (Fig. 3, red cycle). The measured average area also indicated that the lymphoid follicles grew as the BC size increased during the evolutionary phase (Fig. 2).

Special morphological changes in the BC: In many lymphoid follicles, the segregation between the bursal cortex and medulla was not clear or even did not exist on days 1 and 5. In contrast, the cortex containing many lymphocytes

could be distinguished from the lesser dense cellular medulla on days 7 and 9 (Fig. 3C, D, arrows). In particular, the segregating line between the cortex and medulla could be clearly observed on day 9. Fundi of the bursal lumen (Fig. 3B, arrows) showed a cuboidal monolayered epithelium on the lateral side and a multilayered epithelium on the central side of the calyx bordering the medulla in a lymphoid follicle. In addition, many cavities (asterisk) were found in the BC of *Leiostichus leutea*, which constituted a special phenomenon in this study.

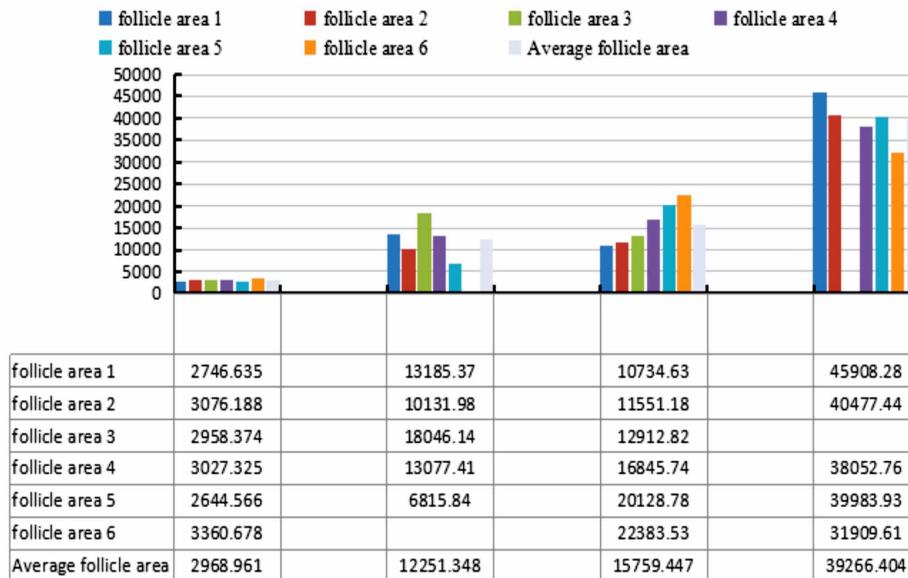


Fig. 2. The size of the lymphoid follicles in the BC. Notes: The magnification time for all statistical data presented above is 10⁴. Follicles 1, 2, 3, 4, 5, and 6 denote the statistical area values.

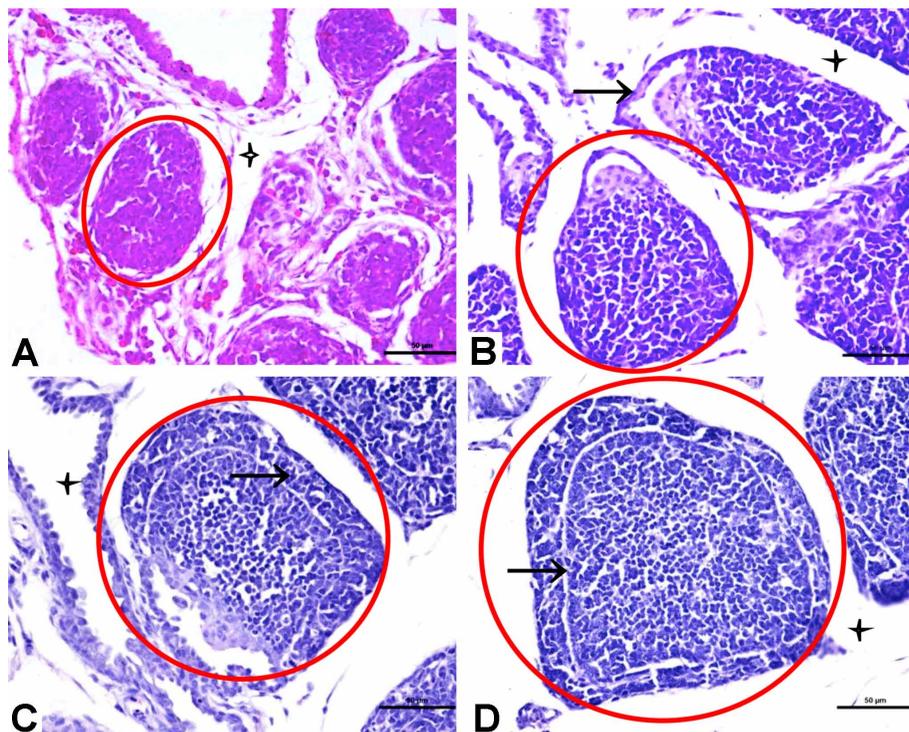


Fig. 3. A. Bursal lymphoid follicles at 1 day of age. B. Bursal lymphoid follicles at 5 days of age (small lumen). C. Bursal lymphoid follicles at 7 days of age. D. Bursal lymphoid follicles at 9 days of age. Tissues were stained with hematoxylin-eosin (HE) and observed under an optical microscope. Scale Bar = 50 μ m.

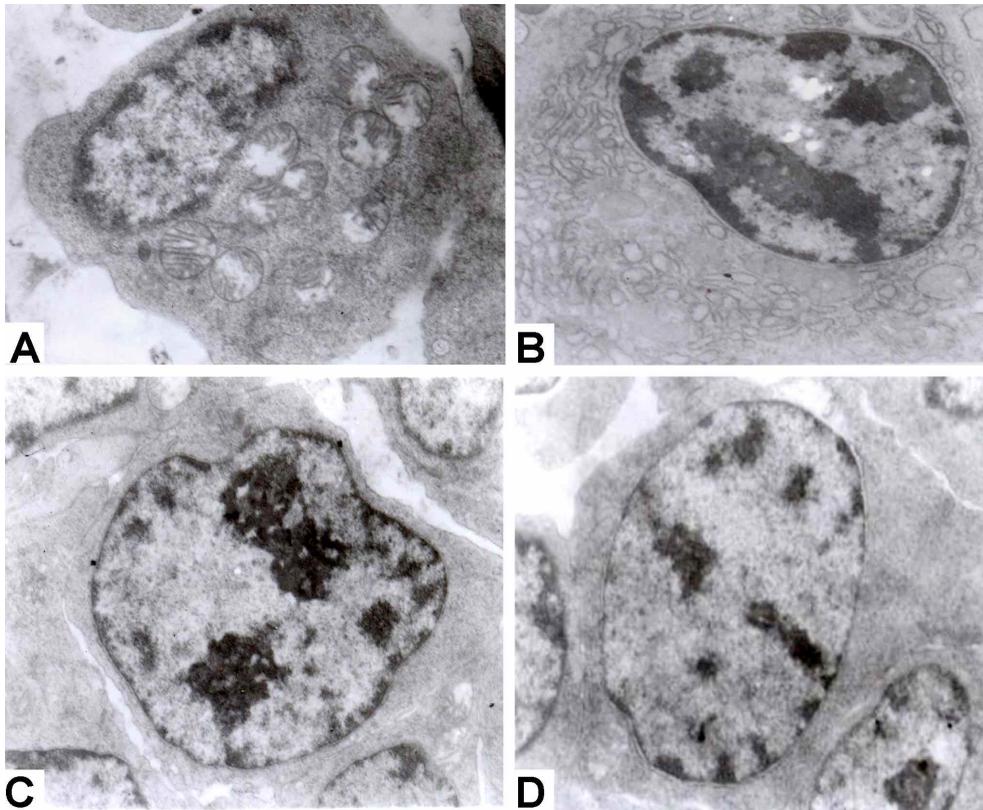


Fig. 4. Differentiation of lymphocytes in the BC.

A: Electron micrograph of 1-day-old bursal cyst lymphocytes shows a large cell body abundant in organelles (10,000 \times).

B: Electron micrograph of 5-day-old bursal lymphocytes shows few organelles and patchy distribution of cell nuclear chromatin (10,000 \times).

C: Electron micrograph of 7-day-old bursal lymphocytes shows clustered distribution of chromatin in the cell nucleus (10,000 \times).

D: Electron micrograph of lymphocytes in the BC at 9 days of age. The nuclei are spherical with less amount of chromatin (10,000 \times).

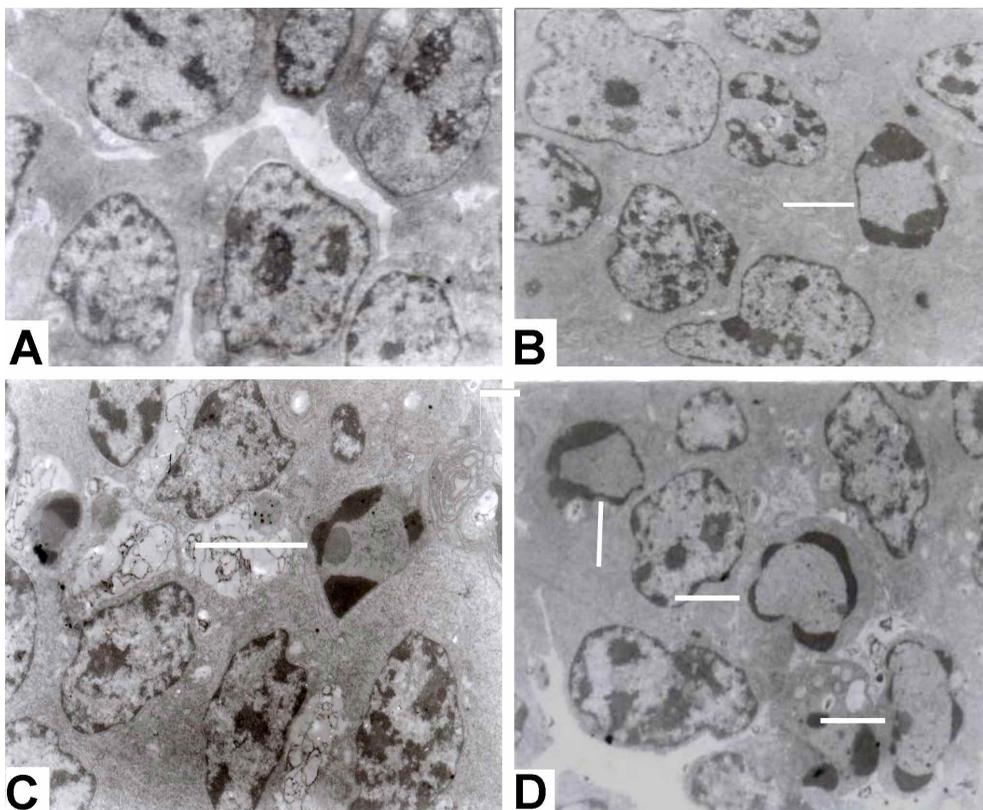


Fig. 5. Cell apoptosis in the BC.

A: Electron micrograph of cells in the BC at 1 day of age shows no apoptotic cells or obvious cell division (7000 \times).

B: Electron micrograph of bursal tissue at 5 days of age shows apoptosis of the individual cells (7000 \times).

C: Electron micrograph of the bursal tissue at 7 days of age shows obvious cell apoptosis, with intercellular lacuna around apoptotic cells (7000 \times).

D: Electron micrograph of cells in the BC at 9 days of age shows a large number of apoptotic cells (7000 \times).

Ultrastructural changes in the BC. Changes in the bursal lymphocytes: The cell bodies and mitochondria of bursal lymphocytes of 1-day-old birds were abundant, and the nucleus, which was small in size, was located on one side of the cell. The nucleus was rich in chromatin, and the cell was in the undifferentiated stage (Fig. 4A). As the days increased, the number of organelles in the lymphocyte decreased and the nuclear volume increased. Some lymphocytes matured and differentiated into plasma cells (Fig. 4B) or effector T lymphocytes (Fig. 4C) at 5 and 7 days of age. At 9 days of age, lymphocytes were fully differentiated with large nuclei (Fig. 4D).

Apoptosis of bursal cells: Figure 5 shows the apoptosis of bursal cells. Shrinking of apoptotic cells and the condensation, fragmentation, and marginalization of the chromatin can be observed clearly. The number of apoptotic

cells (arrows) in the BC increased significantly from days 1 to 9.

The secretion of mucous cells: The secretion of mucous cells in the BC could be observed clearly (Fig. 6). A large number of granular substances were distributed around the cell (arrow), and with the increasing age, the number of particles secreted gradually increased.

Lymphocyte division and intercellular lacunae in the BC: Figure 5A clearly shows the lymphocyte nuclear proliferation and division. The two sets of chromosomes moved towards the pole (arrow), and the cells were in anaphase. As shown in Figure 7B and Figure 5C, some areas where the lymphocytes and apoptotic cells co-exist have lacunae of varying sizes (pentacle). A few chemicals were distributed around the lacunae, and the size of the lacunae increased with increasing age.

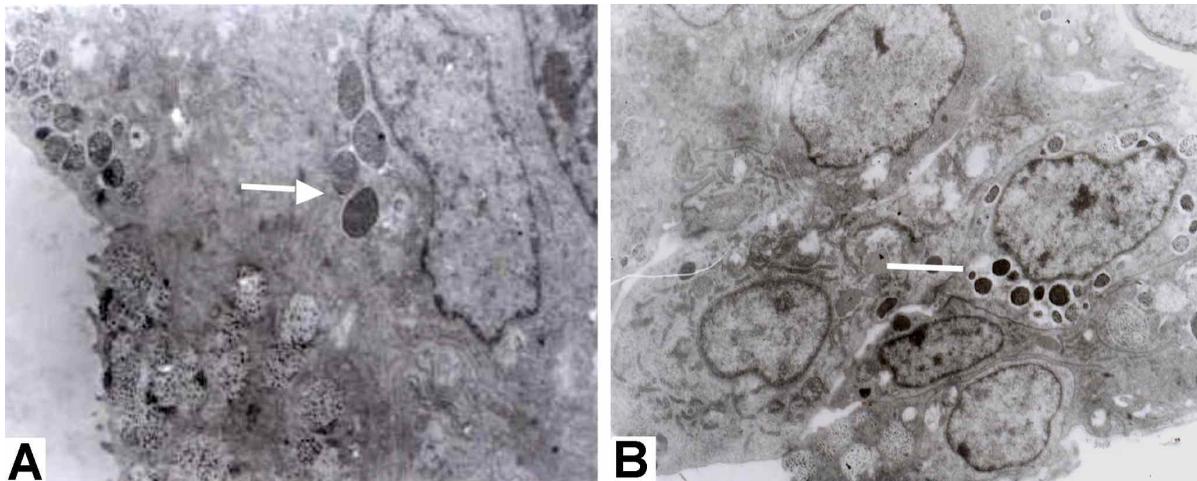


Fig. 6. Lymphocyte secretion in the BC. A: Electron micrograph of the secretory cells in the BC shows clear extracellular secretion (10,000 \times). B: Electron micrograph of the lymphocyte secretion, showing cellular endocrine vesicles (7000 \times).

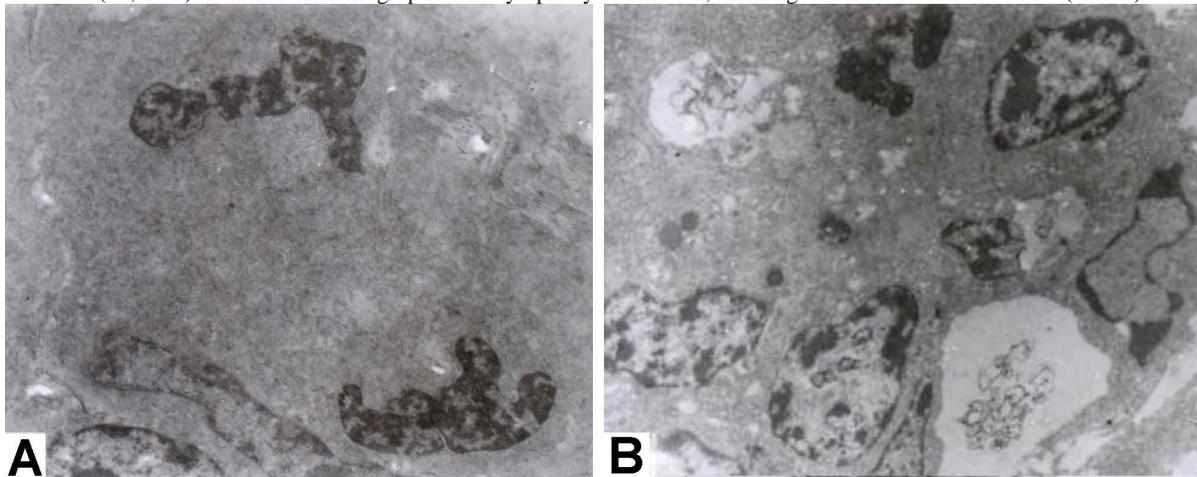


Fig. 7. Lymphocyte division and intercellular phenomena in the BC. A: Electron micrograph showing lymphocyte proliferation in the BC. At this time, the cells are in anaphase, and the chromosomes move towards the cell poles (15,000 \times). B: Electron micrograph of the intercellular space in the BC shows the co-existence of apoptotic cells and intercellular lacunae, and the secretions in the space are clearly visible (7000 \times).

DISCUSSION

The BC of birds has a limited lifespan, which is associated with its function (Hoffmann-Fezer & Lade, 1972; Lupetti *et al.*, 1986; Glick, 1991). In pigeons, three phases of life can be distinguished, namely an evolution phase, a mature phase, and an involution phase. In this study, we focused on the BC of *Leiothrix lutea* at a phase appearing a few days after hatching (i.e., before the evolution phase), with the aim to examine the microstructure and ultrastructure, and the dynamic changes in the BC in this phase (days 1, 5, 7, and 9), because *Leiothrix lutea* is altricial, and the earlier days are very important for its survival in the wild.

Anatomically, the BC of *Leiothrix lutea* is similar to that of pigeons, which is dorsally located on the proctodeal part of the cloaca (Yuan *et al.*, 2015). It takes the shape of a single oval sac with a collum. In this study, we used HE staining and light microscopy to observe the microstructure, and uranyl acetate/lead citrate staining and TEM to observe the ultrastructure of the BC. In addition to the changes in size of the lymphoid follicles as the days increased, we found that the distribution of the follicles was different from that observed in other avian species. The lymphoid follicles distribute evenly across the BC of *Leiothrix lutea*, which is contradictory to that observed in other birds. This structure may be conducive to resist pathogen invasion and related to the environment where *Leiothrix lutea* live. In addition, obvious changes were noted in the structure of the cortex and medulla at the different ages. After hatching, the differentiation of the cortex and medulla was not complete, and the segregating line between the cortex and medulla could not be clearly found and remained nonexistent even at 1 day and 5 days of age. However, the segregation of the cortex and medulla was obvious and a cellular layer on the medullary margin could be noticed clearly at days 7 and 9. However, the cells in this layer presented with large bodies, and loose and orderly arrangement, and there was a huge gap between the cortex and medulla (visible on day 7, obvious on day 9).

The BC is also constituting the microenvironment for maturation and differentiation, and confers immunocompetence to the avian equivalent B lymphocytes (Glick, 1991; Masteller *et al.*, 1997; Ratcliffe, 2002) and T lymphocytes (Fonfría *et al.*, 1994; Cortes *et al.*, 1995). The mature B cells migrate to the peripheral lymphoid organ to settle, reproduce, and perform important functions (Brand *et al.*, 1976; Glick, 1977). In this study, we found that in the early age after hatching, most of the lymphocytes (trachychromatic B cells stained by HE) in the lymphoid follicle were the mature B lymphocytes. The lymphocytes

in this stage present with abundant cytoplasm and mitochondria, which indicated that in this stage, the cells strongly possessed the functions of synthesis, division, and proliferation.

As the age of the birds increases, the number of plasma cells increases, because the B cells are responsible for the host's response to the outer environmental foreign materials (viruses, bacteria, parasites, etc.) and generate antibodies (Cooper, 2015) after hatching, which play an important role in the humoral immunity of birds. During the period that occurs shortly after hatching and extends up to 2 to 4 weeks of age, B cell development depends on antigens that are actively transported from the bursal lumen into the lymphoid compartment of the BC (Ratcliffe, 2006). It has been reported that removal of the BC during the period of rapid growth can diminish the antibody response to *Salmonella* (Taylor & McCorkle, 2009). In birds, the humoral immunity in week 1 of life generally depends on the normal functioning of the BC (Chrzastek *et al.*, 2011). In this stage, the B cells convert into plasma cells. In addition, more apoptotic cells can be observed as the age increases. We speculated that this phenomenon may be related to the external pathogens, such as viruses, bacteria, and parasites. In the early developmental age, cell apoptosis plays a key role in selective differentiation among birds in the wild.

Interestingly, the gross anatomy and microstructure revealed that many cavities were present in the BC of *Leiothrix lutea* for a few days after hatching. This phenomenon may be related to its anti-pathogenic activity, which originates from the gut. Furthermore, numerous independent small cavities were observed in the vicinity of a large cavity, which was formed by the differentiated epithelial cells in the cortex follicles. The lumens attached to these follicles become increasingly large with the increasing age. However, most of the large cavities are located on the bursal ventral side rather than the center, because of which, the density of the surrounding follicles remains low. Contrasting to chicken and other poultry animals, in *Leiothrix lutea*, some follicles at these locations have their own lumen differentiation mechanisms, and theoretically, the lumen is vital to the immune efficiency of *Leiothrix lutea*. These medullary cavities directly interact with the follicles, and hence, we believe these cavities constitute a channel for transferring material and information across the internal and external spaces of the follicle. It is well known that the BC is located between the cloaca and the sacrum, which are connected via the bursal duct to the proctodeum (Madej *et al.*, 2013), as well as the gut-associated lymphoid tissue (Ratcliffe, 2002). Hence, the cavities may play a major role in the anatomical organization of the BC, which has evolved to focus gut-derived antigens from the

bursal lumen towards the lymphoid follicles. A study reported that the chicken BC functions as a peripheral lymphoid organ, wherein specific antibodies are formed against antigens and introduced into the bursal lumen (Hippelainen *et al.*, 1987). In the present study, many secretory granules were observed in the vicinity of large cavities through TEM. Histology demonstrated that the secretory cells in the shape of elongated and short dendrite-like particles were usually located near the junction of the cortical and medullary follicles, and would secrete appropriate materials, such as carbohydrates or protein, according to the needs of host organs and tissues through secretory granules; however, mostly immunoglobulins are the main materials secreted from the gut under antigenic stimulation (Nagy *et al.*, 2004). Kirk *et al.* (2010) reported that when an antigen appears in the final stage of plasma cell development, the rate of immunoglobulin production reaches 3000 /s. Therefore, the structure of the secretory cells and secretory granules in red-billed leiothrix indicated that the immune response was vigorous, even at a young age. At the same time, a large number of plasma cells accumulating in the endoplasmic reticulum suggested that in the early developmental stage, the BC already possessed the ability to generate immunoactive substances and effectively generate the humoral immune response. Thus, we speculated that the cavities were initially tiny and gradually increased in size, interacting with each of the B cells and possibly with the secretory cells or secretory granules, and constituted the place of defense against antigens originating from the gut.

Intercellular lacunae were often found at sites where apoptotic cells and lymphocytes appeared simultaneously (Figs. 4c and 6b). The secretion indicates the difference between the lacuna and intercellular space. Considering the age, we believed that the size of the lacuna was closely related to the development of birds. To date, studies on intercellular lacunae in immune organs are rare. After observing intestinal mucosal cells of Chinese turtle, Chen *et al.* (2005) found many gaps of different sizes between epithelial cells. Ning *et al.* (2004) found that heat stress can increase the intercellular lacuna in the BC of broilers. Therefore, we believe that the lacunae may be a channel for transport of secreted molecules across cells. After hatching, the birds are exposed to the outside environment and the ability of the BC to tackle external antigens is enhanced. Furthermore, with the maturation of the BC, the proliferation and differentiation of lymphocytes is also enhanced. The secretion of immunoactive substances is gradually increased, which forces the expansion of material transport channels across cells. At this point, the increase in cell apoptosis in the BC can be regarded as a self-protection strategy to maintain the stability of its internal environment and the dynamic balance in its cell number (Mondello & Scovassi,

2010). Therefore, apoptotic cells around the cell lacunae may be initiative “suicide” cells in order to maintain a strong host immunity.

As the central immune organ in the brooding period of *Leiothrix lutea*, the BC can produce specific antibodies targeting the antigens entering the sac (Hippelainen *et al.*). In this study, a large number of secretory particles were observed in some lacunae by TEM. This phenomenon indicates that the BC has the ability to produce immunoactive substances and effective humoral immunity at the brooding stage. The increasing ability of secretion of immunoactive substances also indicates that *Leiothrix lutea* has a higher immune function than other birds. At the same time, other immune organs in birds are not fully developed after birth, and hence, the immune response of the BC in this early developmental period is very important for nestlings to fight against exogenous pathogens.

In conclusion, the process of development of the BC in *Leiothrix lutea* is adaptive to its environment. During the initial 9 days after birth, the temperature of the nestlings varies greatly, and the host is highly dependent on the immune system. As an important site of immune response in nestlings, the BC develops to enhance nestling immunity. The proliferation and differentiation of lymphocytes and the secretion of immunoactive substances are the histological manifestations of immune function enhancement. By controlling the apoptosis of some cells in the BC, the number of immune cells and hence, transportation of immunoactive substances can be increased.

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WU, B.; YU, Z.; DENG, J.; YUAN, S. & PENG, XI. Estructura histomorfológica de la *Bursa cloacalis* (Bursa of fabricius) en jóvenes *Leiothrix lutea*: un estudio comparativo utilizando microscopía óptica y microscopía electrónica de transmisión. *Int. J. Morphol.*, 39(6):1646-1654, 2021.

RESUMEN: Este estudio tuvo como objetivo investigar la microestructura y ultraestructura de la *Bursa cloacalis* (BC) en *Leiothrix lutea* joven unos días después de la eclosión, utilizando

microscopía óptica y microscopía electrónica de transmisión. La BC se muestreó a los 1, 5, 7 y 9 días de edad del *Leiothrix lutea*. Inmediatamente después de la disección, se observó la estructura y la integridad de la CB (no degenerativa) y se pudo visualizar con precisión las características temporales específicas. Después de la tinción con hematoxilina-eosina y con acetato de uranilo /citrate de plomo, pudimos observar claramente la microestructura y ultraestructura de la BC. Las observaciones microscópicas revelaron el cambio en el tamaño de la CB o de los folículos linfoides y además, se encontraron numerosas cavidades en la CB; la distribución de los folículos linfoides en *Leiothrix lutea* era diferente a la de otras aves; y la línea de segregación entre la corteza bursal y la médula se hizo cada vez más clara a medida que aumentaba la edad. En conclusión, los datos estructurales obtenidos en este estudio proporcionan una mejor comprensión de la función inmunológica específica de la *Bursa cloacalis* en *Leiothrix lutea*.

PALABRAS CLAVE: *Leiothrix lutea*; *Bursa cloacalis*; **Bursa de Fabricio; Microestructura; Ultraestructura; Folículos linfoides**

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