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Morphological development of the ovary in the *Alectoris chukar* at embryonic and pre-pubertal stages

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Abstract

Alectoris chukar (AC) is a common model organism in biological research. To understand oogenesis and folliculogenesis mechanisms in bird reproduction, we analyzed the ovarian tissue structure of AC at embryonic and pre-pubertal stages. Fertilized eggs, newborn chicks and juvenile AC were used to study the tissue structure of female gonads. Sections of ovaries were prepared and examined using various histological techniques including Hematoxylin and Eosin, Periodic acid-Schiff and Masson's trichrome. Semi-thin and ultra-thin sections of ovary in newly-hatched chicks were prepared for study by electron microscope. The study revealed asymmetry between the left and right ovaries, with a larger left ovary. The functional left ovary exhibited a cortex and medulla, containing somatic and germ cells, with an increase in germ cell number, size and volume leading to cortex thickening. Meiosis division of germ cells and oocyte formation were observed with pre-follicular cells surrounding them. Electron microscopy revealed mitochondria and desmosome cell junctions in germ cells. Our study provided insights into tissue changes in ovaries and germ cells at different developmental stages of AC embryos, newly-hatched chicks and juvenile AC. The results suggested that cortex thickening and germ cell mitochondria density could be used as hallmarks of healthy AC maturity under normal physiological conditions. Further research should explore the impact of growth factors, hormones and environmental factors to unravel avian ovarian development complexities and improve AC reproductive biology knowledge.

Introduction

Birds, specifically chickens, are a valuable model for studying germ cells during embryogenesis due to their unique accessibility inside the egg during various stages of development. This accessibility has made them crucial in understanding vertebrate evolution. The study of gonad development in chickens has provided valuable insights into reproduction, as these organs play a significant role in the production of germ cells. Gonads are specialized organs responsible for the production of gametes (ovum or sperm) and the synthesis of hormones essential for reproduction.[1,2] In female birds, the ovaries are the primary gonads responsible for egg production. The process of ovarian development involves the formation of primordial germ cells, which give rise to oocytes, the female gametes.[3] The differentiation and maturation the female gametes within the ovary are fundamental to the reproductive success of birds. The study of gonad development in avian species has provided valuable information on the mechanisms underlying gamete formation and reproductive function.[4] Histological studies in ostrich and chicken during development has been reported.[5,6] Research on the primary events in gonad and germ cell development has shed light on the unique features of avian reproduction.[4] However, there is still much to learn about the gonad structure in certain bird species including partridges.

Partridges are important in avian reproduction due to their economic value and breeding potential. Understanding the tissue formation of the ovary in the embryonic and pre-puberty stages of partridges can provide insights into their reproductive biology. The diverse species of partridges found worldwide offer a unique opportunity to study the variation in gonad structure and development among avian species.[7]

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The *Alectoris chukar* (AC), commonly known as the Chukar partridge, is a medium-sized game bird native to southern Eurasia and introduced to various regions worldwide for hunting and game purposes. These birds are characterized by their distinctive appearance: Mottled brown plumage, a red beak and striking black and white markings on their faces. Chukar partridges are known for their agility and ability to navigate steep, rocky terrains in their natural habitats which include mountainous regions and arid landscapes. The Chukar partridge main habitat ranges from the mountains of Asia Minor to various regions around the world including North America, New Zealand, and Iran.[8] This widespread distribution of this bird offers researchers the opportunity to study the variation in gonad structure among Chukar partridges from different regions.[9] Furthermore, the dietary habits of Chukar partridges, which include feeding on insects, grasses, plant leaves and fruits, play a crucial role in their reproductive physiology. Understanding how their diet influences gonad development and function can provide valuable insights into optimizing breeding strategies and conservation efforts for this species.[10]

Reproduction studies on Chukar partridges have focused on investigating their breeding behavior and nesting habits.[7] These birds typically form monogamous pairs during the breeding season with males engaging in courtship displays to attract females. The female Chukar partridge constructs a ground nest lined with vegetation where she lays a clutch of eggs, typically ranging from eight to 20 eggs, depending on environmental conditions. Studies on the reproductive biology of Chukar partridges have revealed insights into their egg formation, incubation behavior, and parental care strategies.[10] Moreover, researchers have investigated the hormonal regulation of reproductive processes in these birds, including the role of gonadal hormones in controlling egg production and incubation behavior.[11] Understanding the reproductive physiology of Chukar partridges is essential for optimizing breeding programs and conservation efforts for this species. Studying the reproductive physiology of Chukar partridges, specifically their gonad structure and development, is crucial for advancing our knowledge of avian reproduction. While research on avian gonads has primarily focused on chickens, there is limited information available on the reproductive physiology of Chukar partridges.[12]

Research on the primary events in gonads and germ cell development in Chukar partridges can provide valuable insights into their reproductive biology. Herein, different stages of gonad development were studied in the case of the AC gonad morphological characteristics during their embryonic to maturity development process and to investigate the physiological hallmarks of the normal gonad development characteristics.

Materials and Methods

Samples dissecting and fixation. All experiments were approved by the Ethics Committee of Ferdowsi University of Mashhad (IR.UM.REC.1400.367). Some fertilized eggs of AC were purchased from a breeding center. These eggs were carefully nurtured in an automated incubation machine set at a precise temperature of 37.50 ˚C and a humidity level of 60.00%.

In each embryonic ages, the embryo was dissected and if was female, the left ovary was obtained. In this study, 10, 14, 20-day embryos, newly-hatched chicks and juvenile females AC (three samples from each stage) were used. Under a stereomicroscope (Stemi SV6; Zeiss, Oberkochen Germany), the eggs were opened at the blunt end with a little scissor. Through a delicate process, the embryos were taken from the yolk, meticulously dissected, their gonads were meticulously examined and female gonad was separated. Samples (left ovary) was immersed in Bouin's solution (Merck, Darmstadt, Germany), ensuring optimal fixation for further analysis. Formalin buffer provided another route for fixation with samples being submerged in a 10.00% formalin solution for a minimum of 48 hr. at room temperature. Sex of newly-hatched AC chicks was determined by polymerase chain reaction (PCR). Genomic DNA was extracted from feather samples using DNA extraction Kit (Dena zist Asia, Mashhad, Iran) according to the manufacturer's instructions. Sex of chicks was determined using a primer pair: 2550 F: 5`-GTTACTG ATTCGTCTACGAGA-3` and 2718 R: 5`-ATTGAAATGATCCA GTGCTTG-3` that amplify the homologous part of sex chromosomes (W/Z). For 25.00 µL total volume of master mix, 1.00 µL for each primer (10.00 µM), 12.50 µL of RED master mix (Ampliqon, Odense, Denmark), 3.00 µL of extracted DNA, 7.50 µL distilled water were mixed. Thermal program of PCR was as follows: Initial denaturation of 94.00 ˚C 10 min, 30 cycles of 94.00 ˚C 30 sec, 55.50 ˚C 20 sec, and 72.00 ˚C 30 sec, and a final extraction 72.00 ˚C 5 min. PCR products were electrophoresed on agarose gel and visualized under ultraviolet irradiation. Two bands indicated female (W/Z chromosome) and one band showed male chicks (Z/Z chromosome). In birds, the sex chromosomes are designated as W and Z, and females are heterogametic (WZ) while males are homogametic (ZZ).[13] Regarding, female newly-hatched chicks and female juvenile females AC after decapitation of chicks and the drainage of blood, the left ovary was anatomically analyzed and separated from the body and placed in fixative solution.

Light microscopy. Sample preparation for producing normal sections involved fixing the samples, dehydrating them in ethanol solutions of increasing concentrations (70.00, 80.00, 90.00, and 100%) and cleaning them in xylene, embedding them in paraffin (Merck) and then cutting 5.00 µm sections with a microtome (RM 2145;

Leica, Wetzlar, Germany). The prepared sections were stained by the following methods: Hematoxylin and Eosin, Periodic acid-Schiff (Merck) and Masson's trichrome (Merck) and images were collected using a light microscope (BX51; Olympus, Tokyo, Japan) equipped with a digital camera (DP12; Olympus).

Semithin sections and electron microscopy. For semi-thin and ultra-thin sections three left ovaries of newly-hatched chicks were used. Samples were bathed in a 2.00% glutaraldehyde solution, then re-fixed in 1.00 % osmium tetroxide in 1.00 M cacodylate buffer, after that gradually dehydrated in ethanol and embedded in epoxy resin (TAAB Laboratories Equipment Ltd., Aldermaston, UK) and cut in 1.00 µm thickness (semi-thin sections). The semi-thin sections were stained with toluidine blue. For ultra-thin, 80.00 nm thickness sections were prepared from resin blocks using an ultra-microtome (Leica Microsystems, Vienna, Austria). Ultra-thin sections were stained with uranyl acetate and lead citrate. The latter sections were observed with a transmission electron microscope (LEO 912AB; Zeiss).

Results

Macroscopical observations of the ACs' ovaries. The ovaries were attached in the abdominal cavity upper part and on the mesonephro ventral-middle surface (Figs. 1A and B). In a 20-day-old AC embryo, the asymmetry of the right and left ovaries was observed and in newly hatched chicks the left ovary was larger than the right one.

Ten-day-old ACs' embryo. The mesonephros kidney a transient embryonic structure that eventually gave rise to the permanent kidneys was observed situated under the ovaries. Therefore, it was shown that ovary was detectable in the ten-day-old AC embryo. In the current work, in the transverse sections, the ovary was oval in shape (Figs. 1A and 1B). Furthermore, the thin layer of the cortical tissue, comprising the dense and loose connective tissues, was seen enveloping the mesonephros kidney. Beneath this cortical layer, a distinct portion of the medulla was visible comprising the renal pyramids and their surrounding interstitium (Figs. 1A, 1B and 1C).

Fourteen-day-old ACs' embryo. At this age, the gonads exhibited a distinct morphology characterized by a noticeably thicker outer layer commonly referred to as the cortex. This cortical layer was comprised of a tightly packed arrangement of cells which appeared denser compared to previous days. Beneath this cortical layer, the medulla was visible, featuring a distinctive pattern of organization (Fig. 1B). The medulla, in turn, was composed of a meshwork of mesenchymal cells which were dispersed throughout the layer. These mesenchymal cells, often appearing as spindle-shaped or stellate cells, were readily observed within the medulla layer. The AC embryos ovary showed a poor reaction with the Periodic acid-Schiff staining (Fig. 1C). Also, the amount of collagen fibers was less except between the cortex and medulla, tunica albuginea which collagen fibers were more there (Fig. 1B).

Twenty-day-old ACs' embryo. In this age, two distinct parts of the cortex and medulla were observed. The outer margin of the ovary was characterized by the germinal epithelium. The cortex itself comprised several layers of somatic pyramidal cells. Additionally, the cortex contained germ cells, which were larger and more brightly stained than the surrounding cells due to their increased nuclear density and proliferative activity (Fig. 2A). The medulla, on the other hand, was a compact somatic cell cords separated by mesenchyme cells which provided structural support and guidance to the developing ovarian tissue. Within the medulla of the ovary, several specialized structures were identified, including lacunar canals. The medulla also contained mesenchymal cells and interstitial cells (Figs. 2A and 2B). Furthermore, the medulla was richly vascularized.

Newly-hatched chick of AC. In newly hatched chicks, the ovary was observed to comprise two distinct regions, the cortex and the medulla (Fig. 2C). The cortex, situated on the outer surface of the ovary, was characterized by the presence of a germinal epithelium, a layer of cells that gave rise to the formation of secondary sex cords (Fig. 3A).

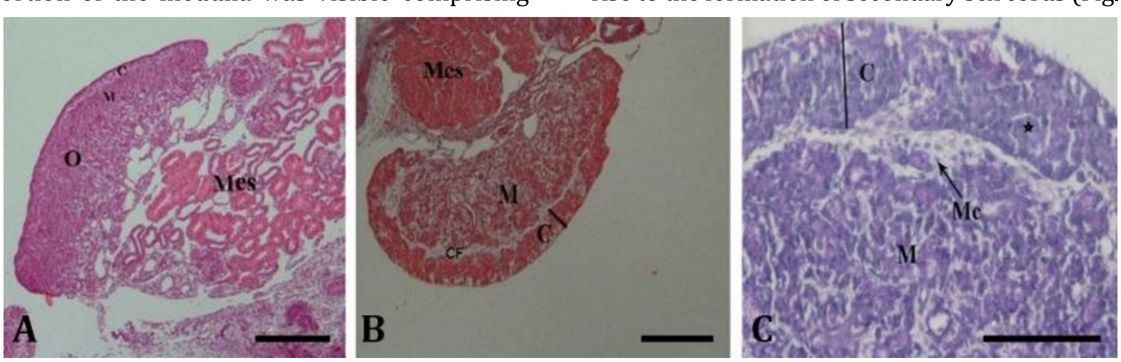

Fig. 1. A and **B)** Histological structure of the left ovary in 10-day-old and **C)** 14-day-old AC embryos indicates: ovary (O), the thin cortex (C), medulla (M), mesonephros (Mes), collagen fibers (CF), mesenchymal cell (Mc) and carbohydrate (asterik). **A)** Hematoxylin and Eosin staining; bar = 200 µm), **B)** Masson's trichrome staining; bar = 200 µm, **C)** Periodic acid-Schiff' staining; bar = 100 µm.

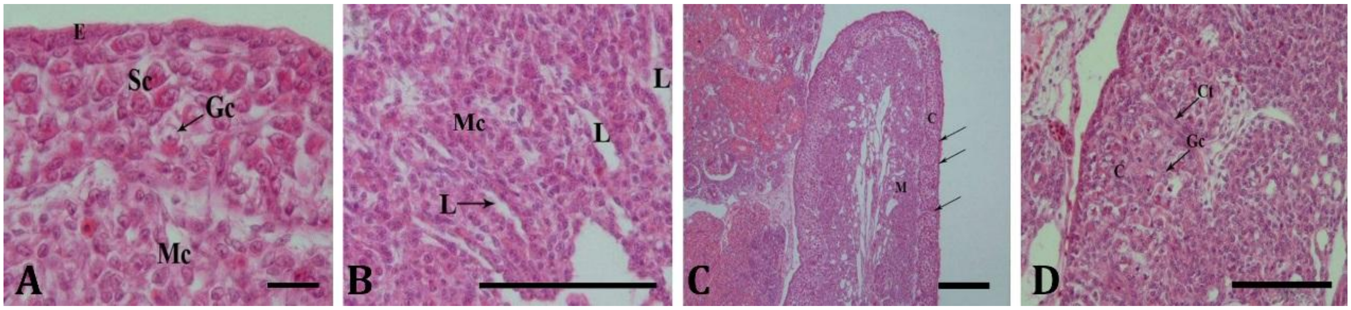

Fig. 2. A and **B)** Histological structure of the left ovary in 20-day-old AC embryos, **C** and **D)** newly-hatched chick of *Alectoris chukar*. Ovary with well-defined cortical (C) and medullary (M) parts and a layer of mesenchymal cells (Mc) located in the medulla. Somatic cells (Sc) and germ cells (Gc) are seen in the cortex and Mc and the lacunar canals (L) in the medulla. The outer layer of the cortex contains cells of the germinal epithelium (E). Also, the penetration of connective tissue into the cortex layer is shown by arrows and the connective tissue infiltrated into it (Ct), as well as the Gc in this layer that begin meiotic division. (Hematoxylin and Eosin staining; bars = 20.00, 100, 200, and 100 µm, respectively).

The germ cells, which were accompanied by somatic cells, were responsible for the development of these cords. The boundary between the cortex and the medulla was demarcated by a thin layer of connective tissue, which not only separated the two regions but also penetrated the cortex, giving it a lobed appearance (Fig. 2D). Furthermore, a notable difference was observed between the cortex of the newly hatched chicks and its embryonic state. In comparison, the cortex had become significantly thicker and many germ cells within it had initiated meiosis (Figs. 2D and 3A).

Semi-thin sections and electron microscopic study in newly hatched chick. An electron microscope study of the left ovary revealed the presence of organelles including mitochondria in germ cells. In the nucleus of germ cells, dense masses of chromatin were observed. Surrounding the germ cells, pre-follicular cells were present which eventually coalesced to form the follicle in the future along with the oocyte (Fig. 3C). Also, in semi-thin sections germ calls, nuclear chromatin, somatic cell, germinal epithelium, erythrocyte and lacunar channels were observed (Figs. 3A and 3B). Furthermore, in ultra-thin sections vacuoles in germ cells and desmosome junctions between cells were distinct (Fig. 3D).

Histological structure of ovary in juvenile AC. In the ovarian sections of the juvenile partridge, several follicles were observed within the ovary. These follicles contained oocytes at various stages of development. The oocytes were surrounded by a layer of granulosa cells. The follicular fluid was surrounded by a basement membrane which separated the follicle from the surrounding ovarian tissue (Figs. 4A and 4B).

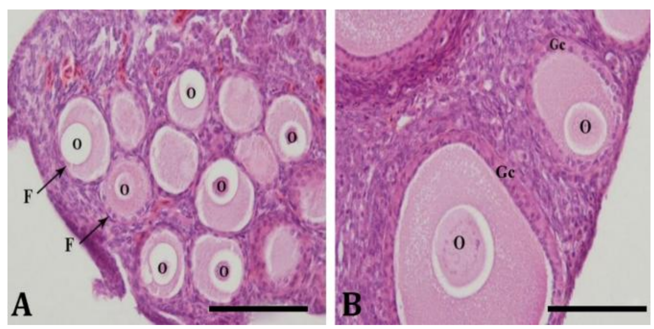

Fig. 4. Histological structure of ovary in juvenile quail. **A)** Microscopic image of the left ovary with a large number of follicles (F) and oocytes (O). **B)** A microscopic image of the left ovary shows the oocytes (O) inside each follicle and the granulosa cells (GC) around it (Hematoxylin and Eosin staining, bars = 100 µm).

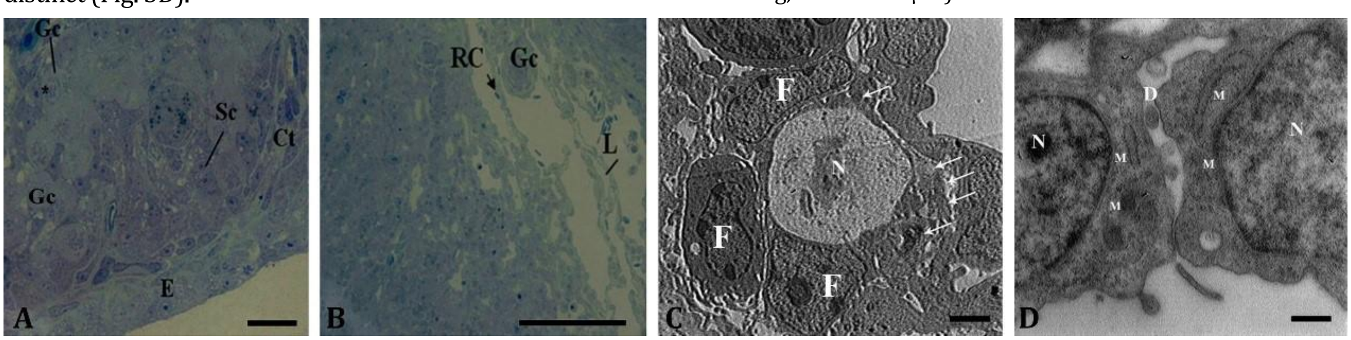

Fig. 3. A and **B)** Semi-thin sections of the cortex and medulla using Toluidine blue staining, **C** and **D)** transmission electron micrographs of the left ovary of the newly-hatched chick of *Alectoris chukar*. Germ cells (Gc), somatic cells (Sc), connective tissue (Ct), germinal epithelium (E), follicles (F), red blood cell (RC) and lacunar canals (L). The light-colored germ cell, where the nucleus (N) and mitochondria (marked by M and arrows) are marked. The image indicates the connection of germ cells is of the desmosome type (D). Bars = 20.00 µm, 100 µm, 2.00 µm, and 500 nm, respectively.

Discussion

The development of the ovary is a complex process that involves multiple stages and cellular interactions. From an early embryonic stage, it is possible to observe asymmetry between the right and left ovaries. The anatomical asymmetry becomes macroscopically evident at stage 34 (E 8 day) in chicken ovaries. As development progresses, distinct regions within the ovary emerge including the cortex and medulla.[5,6]

The cortex is characterized by a germinal epithelium which serves as a critical site for the formation of ova and gives rise to secondary sex cords. The germ cells are responsible for the development of these cords. While the medulla was composed of compact somatic cell cords separated by mesenchyme cells. Similar results have been reported in chicken and ostrich[5,6]. Somatic cells in cortex and medulla are responsible for the maintenance and differentiation of the ovary tissue. Mesenchymal cells are precursors to connective tissue and play a crucial role in shaping the ovary architecture. Blood vessels of medulla supplying essential nutrients and oxygen to the developing ovarian tissue.[14]

The presence of narrow lacunar canals within the medulla facilitates cell migration and molecule transport during development. Interstitial cells, also present in the medulla, are responsible for producing hormones that regulate ovarian function and fertility. Morphological transformation in ovary of newly hatched chicks indicates a significant advancement in the development of the ovary as the chick prepares for its reproductive life. In newly hatched chicks, it was observed that many germ cells-initiated meiosis, indicating a significant advancement in ovarian development. This was consistent with previous studies showing that meiosis was initiated in the developing ovary during the early stages of embryonic development.[15]

The existence of follicles in the juvenile partridge ovary was also consistent with previous studies. The development of follicles is a critical step in the maturation of the ovary as they provide a means for oocytes to grow and mature. The presence of granulosa cells surrounding the oocytes suggests that these cells play a crucial role in providing nutritional support and regulating growth and maturation. The granulosa cells also formed a follicular fluid which is a clear liquid that bathes the oocytes and granulosa cells providing them with essential nutrients and hormones. Vacuoles which are present in immature follicles gradually mature and are revealed to be yolk vacuoles. This indicated the initiation of follicular development and the accumulation of yolk material necessary for oocyte maturation.[16]

The electron microscope study provides further insight into the ultrastructure of germ cells during embryonic development. The presence of multiple mitochondria in germ cells suggested that these cells were in a high metabolic function. The dense masses of chromatin observed in the nucleus of germ cells were also consistent with previous studies showing that these cells were actively transcribing and replicating their genetic material.[17] Dense masses of chromatin in the nucleus of germ cells exhibited a similar arrangement to that seen during the mitotic stage. This arrangement was indicative of the active transcription and replication of genetic material necessary for the development of the embryo.[18]

The results of this study provided valuable insights into the development of the ovary during embryonic and post-embryonic stages. The emergence of distinct regions within the ovary, including the cortex and medulla, suggested a complex process of cellular differentiation and organization. The presence of follicles and granulosa cells in the juvenile partridge ovary indicated a critical step in the maturation of the ovary.

Future studies could focus on elucidating the molecular mechanisms involved in ovarian development including the signaling pathways and transcription factors that regulate cellular differentiation and organization. Additionally, studies could investigate the role of hormones in regulating ovarian development and function. Furthermore, it would be interesting to investigate whether similar developmental patterns are observed in other avian species or whether there are any differences in ovarian development between different species. It would also be useful to study the expression of specific genes involved in ovarian development using techniques such as quantitative PCR or RNA sequencing. This would provide valuable insights into the molecular mechanisms involved in this process.

Finally, it would be interesting to investigate whether there are any differences in gonadal development between males and females or whether there are any differences in ovarian development between different populations or environments.

The present study has unveiled a fascinating asymmetry between the left and right ovaries with a larger left ovary exhibiting a more complex morphology. The functional left ovary showed a clear cortical-medullary structure comprising somatic and germ cells which is essential for the proper development and function of the ovary. Notably, the increase in germ cell number, size and volume led to the thickening of the cortex layer, an age-dependent phenomenon, indicating a critical role of developmental stage in ovarian morphology. The observation of the meiosis division of germ cells and the formation of oocytes, surrounded by pre-follicular cells, provided valuable insights into the early stages of ovarian development in ACs. Electron microscopy revealed the presence of mitochondria and desmosome cell junctions in germ cells highlighting the importance of these cellular structures in maintaining the health and function of ovarian cells. The morphological characteristics of germ cells including their size, shape and density could be used as indicators of ovarian maturity and health.

The study findings provided a comprehensive understanding of tissue changes in the ovaries and germ cells across different developmental stages of AC embryos, newly hatched chicks and juvenile AC. The results had significant implications for the study of avian reproductive biology and might contribute to the development of new diagnostic tools and strategies for improving reproductive success in AC. The study findings also underscored the importance of considering developmental stage and morphological characteristics in the study of avian reproductive biology. Overall, this study provided a fundamental understanding of the physiological processes underlying AC gonad morphology during their development stages, which has significant implications for our understanding of avian reproductive biology and its application to animal breeding and conservation programs.

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Conflict of interest

The authors declare no conflict of interest.

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