Germ cell dynamics during nest breakdown and formation of the primordial follicle pool in the domestic turkey (*Meleagris gallopavo*)

G. B. Hall,[*] J. A. Long,[†] B. J. Wood,[*,‡,§] and G. Y. Bedecarrats[*,1]

*Department of Animal Bioscience, University of Guelph, Guelph, Ontario N1G1Y2, Canada; [†]Animal Biosciences and Biotechnology Laboratory, Beltsville Agricultural Research Center, Agricultural Research Service, USDA, Beltsville, MD 20705; [‡]Hybrid Turkeys, Suite C, Riverbend Drive, Kitchener, Ontario N2K3S2, Canada; and [§]School of Veterinary Science, The University of Queensland, Gatton, QLD, 4343 Australia

ABSTRACT This study determined, for the first time, the different subpopulations of germ cells and stereological changes within the cortex of the functional left ovary during germ cell nest breakdown, and formation of the primordial follicle pool in the domestic turkey. This was accomplished by measuring the size, density, and count of prefollicular germ cells and primordial follicles in turkey poults between 1 and 35 days posthatch (**dph**). The percent volume (**PV**) of germ cells and follicles within the cortex was also calculated as a means of validating the counting technique. The total percent volume of germ cells and primordial follicles within the cortex ranged between 42 and 84%, suggesting that the counting technique was valid. Our findings show that before germ cell nest breakdown (5 dph), there were roughly 1,000,000 prefollicular germ cells within the cortex of the left ovary and that germ cell nest breakdown initiated between 5 and 7 dph, characterized by a decrease ($P \leq 0.001$) in prefollicular germ cell density and the subsequent appearance of primordial follicles. Nest breakdown is followed on day 9 by the first increase ($P \leq 0.05$) in size of prefollicular germ cells. These cells continue to grow throughout nest breakdown. The majority (>90%) of germ cell nest breakdowns concluded by 15 dph; although, the primordial follicle pool was not fully established until 35 dph, as determined by a total lack of prefollicular germ cells. At this point, the pool was comprised of an estimated 60,000 primordial follicles and shows that during nest breakdown and follicle pool formation, ∼94% of germ cells were lost. This 94% decrease in the number of germ cells during nest breakdown in the turkey is comparable to the domestic chicken but is greater than the average two-thirds which are lost in mammalian species.

Key words: Prefollicular germ cell, primordial follicle, nest breakdown, ovary, turkey

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INTRODUCTION

Within the immature avian ovary, the breakdown of germ cell nests, germ cell loss, and formation of primordial follicles marks a pivotal cellular depletion and reorganization; as birds lack a germinal bed with self-renewing germ cells (oogonium) upon sexual maturation (Johnson, 2015). This means that the cellular depletion heavily influences the finite number of ova available throughout a female bird's reproductive life span. Once the follicle pool is exhausted or below a specific threshold, the bird would theoretically enter reproductive senescence.

In mammals, germ cell nest breakdown is well characterized by (1) dissolution of intracellular bridges; (2) an abrupt decline in the number of germ cells predominantly through apoptosis; (3) and infiltration of pregranulosa cells to surround the remaining primary oocytes and form primordial follicles (Pepling and Spradling, 2001; Felici, 2005; Rodrigues et al., 2009; Tingen et al., 2009; Findlay et al., 2015; Wear et al., 2016). The primordial follicle pool is established after the completion of this process. Germ cell nest breakdown is not well characterized in avian species. The series of cellular events is assumed to be consistent with that of the mammalian process. One defining difference is that this process in mammals

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[1]Corresponding author: gbedecar@uoguelph.ca

has been shown to occur before or after parturition; however, in birds, it has only been documented posthatch.

In the domestic chicken, germ cells at the time of hatch are classified as either (1) oogonia which are mitotically active or resting or (2) primary oocytes which have entered meiosis and are paused at prophase I (Hughes, 1963), with a distribution of 30% oogonia and 70% primary oocytes (Gonzalez-Moran, 2007). These germ cells are organized into nests connected by intracellular bridges (Skalko et al., 1972; Ukeshima and Fujimoto, 1991), also known as ring canals. It is assumed that, similarly to mammals, germ cells in the same nest are clonally derived (Wartenberg et al., 1998) and that the intracellular bridges are derived from incomplete cytokinesis when oogonia divided synchronously (Pepling and Spradling, 1998). Germ cell nest breakdown initiates at 3 days posthatch (**dph**) for quail (Madekurozwa, 2012), 4 dph for chicken (Hughes, 1963), and 7 dph for the turkey (Liu et al., 2017), as noted by the first appearance of primordial follicles. To date, germ cell numbers during nest breakdown in poultry species have only been documented using the chicken, and no wild bird studies have been performed. It was reported that the total germ cell population in chickens peaks on day 17 of incubation, before hatch, at around 680,000 (Hughes, 1963), with estimates from 175,000 to 480,000 germ cells on the day of hatch (Hughes, 1963; Méndez-Herrera et al., 1998; Gonzalez-Moran, 2011). It was further reported that germ cell numbers decreased to 75,000 by 7 dph, concomitant with the appearance of primordial follicles and that the follicle pool was fully established by 4 wk of age with a germ cell population of 50,000 (Gonzalez-Moran, 2011). Thus, it appears that in chickens between 70 and 90% of germ cells are lost during nest breakdown and primordial follicle formation. For the turkey and quail, the dynamics, duration, and number of germ cells during nest breakdown and the timing of primordial follicle pool establishment are not known.

The 2 studies presented here aimed to provide fundamental information with regards to germ cell nest breakdown and formation of the primordial follicle pool in the domestic turkey. In the first study, the focus was on determining the size, density, and percent volume (**PV**) of germ cells in the left ovarian cortex during the first 5 wk posthatch. This allowed us to validate our counting technique and identify key time points during nest breakdown and pool formation. Therefore, in our second study, we were able to determine the specific populations of germ cells and follicles before, during, and after germ cell nest breakdown.

MATERIALS AND METHODS

Animals and Sampling

Day-old female poults from a female parent-stock line, supplied by Hybrid Turkeys (Kitchener, Ontario, Canada) were housed in groups at the University of Guelph's Animal Care Facility and given food and water *ad libitum*. The lighting protocol was as follows: 23 h light:1 h dark at 1 dph, followed by a step-wise decrease of 1 h light per day until an 18 h photoperiod was reached at 6 dph. Poults were euthanized by cervical dislocation between 1 and 35 dph with the age of poults defined as being dph ±6 h, based on the hatch window. After euthanasia, the abdomen was opened, and the ovary located on the left dorsal wall was removed and collected. This research was approved by the Institutional Animal Care Committee and adhered to the principles described by the Canadian Council on Animal Care.

Experimental Design

For study 1, left ovaries were collected from 4 poults at 1, 3, 5, 7, 9, 11, 13, 15, 21, 28, and 35 dph (n = 4 per age). The ovaries were then placed in a petri dish containing holding media (PBS with 20% fetal bovine serum). The ovaries were then cut into 2 × 2 mm pieces (3-5 pieces/ovary based on the overall size), with the aid of a dissecting microscope, before processing (Liu et al., 2017). This made tissue sectioning and analysis more efficient. These ovaries were used to determine germ cell and follicle diameters, densities, and PV within the cortex. In study 2, left ovaries were collected from 4 poults at 5, 9, 15, and 35 dph (n = 4 per age), but this time ovaries were kept whole. These ages were identified from study one's results, as key time points before, during, and after germ cell nest breakdown and establishment of the primordial follicle pool. These ovaries were used to determine germ cell and follicle counts.

Tissue Processing and Imaging

Dissected and whole ovaries were fixed in Bouin's solution (water 81.6%, formaldehyde 9.2%, acetic acid 4.9%, methyl alcohol 3.7%, picric acid 0.6%) for 1 h at room temperature and then for an additional 23 h at 4°C (Liu et al., 2017). After fixation, tissues were washed with PBS, transferred into tissue cassettes, and stored in 70% ethanol at 4°C until further processing. All tissues were processed in a Shandon Excelsior ES (ThermoScientific, Cheshire, UK). For dissected ovaries, the protocol involved 6 consecutive cycles of isopropanol (70, 85, 90, 95, 100, and 100%), followed by 3 cycles of xylene (100%) and ending with 2 cycles of paraffin wax (60°C–62°C). Each cycle lasted 30 min. A similar process was used for whole ovaries, except a third cycle was added for paraffin wax, and all cycles were extended to 45 min. All tissues were immobilized in paraffin blocks for sectioning. Dissected ovaries were positioned in blocks to allow all pieces from the same ovary to be sectioned concurrently; whereas, whole ovaries were positioned so that sectioning occurred in a posterior to anterior direction. Tissues were sectioned at a thickness of 5 μm using a Finesse ME microtome (ThermoShandon, Cheshire, UK). In the case of dissected ovaries, 4 serial sections were collected per slide with 100 μm (20 sections) discarded between slides, and 8 to 10 slides were collected per dissected ovary. In the case of whole ovaries, 4 serial

sections were also collected per slide. With varying numbers of sections discarded between slides, based on the age of the ovary: 10 sections (5 dph), 14 sections (9 dph), 20 sections (15 dph), and 34 sections (35 dph), and slides were collected throughout the entire ovary. The difference in discarded sections meant that, on average, the number of slides collected from an ovary at different ages was roughly equal, ensuring larger ovaries from later ages were not oversampled compared with smaller ones (Gonzalez-Moran, 2011). Slides were stained with hematoxylin and eosin and examined under a light microscope, Leica DM 5000B (Leica, Wetzlar, Germany) equipped with a B-Series LED light source (ScopeLED, Richmond, CA) for sequential RGB imaging. The first section on each slide from dissected and whole ovaries were captured using a Hamamatsu Orca-Flash 4 camera (Hamamatsu Photonics, Hamamatsu City, Japan). Images were captured (mag. 40x) using Volocity (ver. 6.3.1; Quorum Technologies, Guelph, ON, Canada) over the entire first section; these images were then subsequently stitched together to provide a large, high-resolution image of the first section. All chemicals were purchased through Fisher Scientific (Hampton, NH).

Histological Examination and Stereology

Nomenclature and Morphological Classifications

The literature does not contain consistent nomenclature regarding the differentiation between a primary oocyte not yet surrounded by granulosa cells to one incorporated into a primordial follicle. It has been previously proposed that germ cells can be classified as either prefollicular or follicular (Felici, 2004), but no specific naming scheme has been proposed. For this study, the nomenclature "prefollicular germ cell" will refer to oogonium and primary oocytes not yet incorporated into primordial follicles.

Morphological classifications were made in accordance with the following publications: Greenfield, 1966; Rothchild, 2003; Gonzalez-Moran, 2011; Liu et al., 2017. For ovaries between 1 and 9 dph, the cortex was defined by the germinal epithelium (external) and a clear differentiation between germ cell nests and medulla (internal). For older ages (11–35 dph), the internal differentiation was lost; therefore, the internal cortex border was defined by the simple presence of primordial follicles and/or prefollicular germ cells. Prefollicular germ cells with the following characteristics were measured and counted: (1) large cytoplasm enabling it to be distinguished from neighboring somatic cells; (2) clear visible nucleus; and (3) intact plasma membrane. Primordial follicles (primary oocytes surrounded by one layer of granulosa cells) meeting the following criteria were measured and counted: (1) one primary oocyte with a clear nucleus, in the center; (2) surrounded by a single defined follicular epithelium layer; and (3) with a clear basement membrane.

Germ Cell and Follicle Diameter Diameter measurements of prefollicular germ cells, primordial follicles, and their primary oocyte within were obtained from the high-resolution images from the dissected ovaries. From each ovary, a maximum of 120 prefollicular germ cells were randomly selected from the 8 to 10 high-resolution images. Height and width measurements were taken and averaged to give the respective diameters (Liu et al., 2017). Similarly, a maximum of 30 primordial follicles and their respective primary oocytes within were measured. All prefollicular germ cell, primary oocyte, and primordial follicle diameters were averaged per ovary. In addition, the smallest (Min.) and largest (Max.) prefollicular germ cells, primordial follicles, and primary oocytes within each dissected ovary were compared to illustrate size range.

Germ Cell and Follicle Density To calculate the density of prefollicular germ cells and primordial follicles within dissected ovaries, the number and cortex volume they occupied were determined (Beck et al., 2018). For prefollicular germ cells, 6 high-resolution images were used per ovary, and the number of prefollicular germ cells within all 6 images were counted and summed together. For primordial follicles, 4 slides (4 serial sections per slide) were used per ovary, and the number of primordial follicles within all 4 serial sections was determined. This was accomplished by using the high-resolution image of the first section of each slide as a reference to ensure follicles were not counted twice in further serial sections, on the same slide (Beck et al., 2018). Primordial follicle counts per ovary were then summed together. Four serial sections were analyzed because of the size increase of primordial follicles when compared with prefollicular germ cells. Only using the first section would have resulted in a gross overestimation of the number of primordial follicles. To determine cortex volume, the perimeter of the cortex was drawn using the "freehand tool" of Volocity, allowing for the area to be calculated. The area was then multiplied by the cortex thickness either 5 μm, in the case of prefollicular germ cells or 20 μm for primordial follicles to give the specific volume of the cortex examined. All respective cortex volumes were summed together. The total number of prefollicular germ cells or primordial follicles were divided by their respective total cortex volume to give their density per ovary.

Percent Germ Cell and Follicle Volumes The PV of prefollicular germ cells, primordial follicles, and the combined total within the individual cortexes of dissected ovaries were calculated using the equation: $PV = \frac{4}{3}\pi r^3 \times D \times 100$ with (r) corresponding to the cell or follicle radius (mm), that is half the diameter, and (D) the density $(\#/\text{mm}^3)$, from each of the dissected ovaries (Beaumont and Mandl, 1961; Baker, 1963; Ioannou, 1964).

Cortex Volume, Germ Cell, and Follicle Counts

The cortex volume within whole ovaries at 5, 9, 15, and 35 dph was determined by tracing the periphery

of the cortex in all the first sections on each slide using the high-resolution images. The area calculated by Volocity was then multiplied by the thickness (μm's) of the sections collected and discarded between the first sections: 70 μm (5 dph), 90 μm (9 dph), 120 μm (15 dph), and 190 μm (35 dph). All volumes per ovary were summed together to give the total cortex volume per whole ovary.

To determine prefollicular germ cell, primordial follicle, and the total germ cell count within whole ovaries, densities were first calculated in a similar manner as explained earlier for dissected ovaries. The densities were then multiplied by the cortex volume per ovary to calculate counts, with total germ cell count being the sum of prefollicular germ cell and primordial follicle counts (Gonzalez-Moran, 2011).

Statistical Analysis

Statistical analyses were performed using SPSS 25.0 for Mac (SPSS Inc., Chicago, IL). Data were presented as means ± standard deviation or standard error of the mean. Normality and equal variance of data were evaluated by residual plots and Levene's tests, respectively, before final analysis. A one-way ANOVA was used to analyze the variance in diameter, density, percent volume, cortex volume, and count, among age groups. Differences were considered as significant when $P \leq 0.05$. If there was an age effect, post-hoc tests (Tukey) were performed to determine which ages differed significantly ($P \leq 0.05$).

RESULTS

General Histology

At early ages (1–5 dph), the cortex was distinguished from the medulla based on clear uniformity of the prefollicular germ cells within (Figure 1A). Germ cell nests within the cortex can be partially identified based on the distance separating them and the presences of immature granulosa cells between them. During the older ages (7–35 dph), when germ cell nests had broken down and individual germ cells were incorporated into primordial follicles, the outer most primordial follicle or prefollicular germ cells were used as references to distinguish the cortex from the medulla (Figures 1B–1D).

During early ages, prefollicular germ cells with a relatively large nucleus and cytoplasm (compared with immature granulosa cells) comprised the majority of the cortex (Figures 2A–2C). This made it impossible to clearly determine individual germ cell nests. Separation between nests was only possible when immature granulosa cells were present between nests. There was an abrupt change in the appearance of the cortex between 5 and 7 dph (Figures 2C, 2D), with an increase in the number of immature granulosa cells loosely surrounding the prefollicular germ cells. By 9 dph, the primordial follicles which had formed had a single epithelial layer of granulosa cells, but these cells were not always cuboidal, instead, they often appeared flattened or squamous (Figures 2E, 2F). At 15 and 21 dph, the primordial follicles were consistently surrounded by the typical cuboidal granulosa cells with their peripheral side defining the basal lamina (Figures 2G–2I). The later 2 time points (28 and 35 dph) showed increased blood vessels within the cortex, and the primordial follicles, still within the cortex, appeared to be forming their first external theca cell layer, showing initial progression toward becoming primary follicles (Figures 2J–2L).

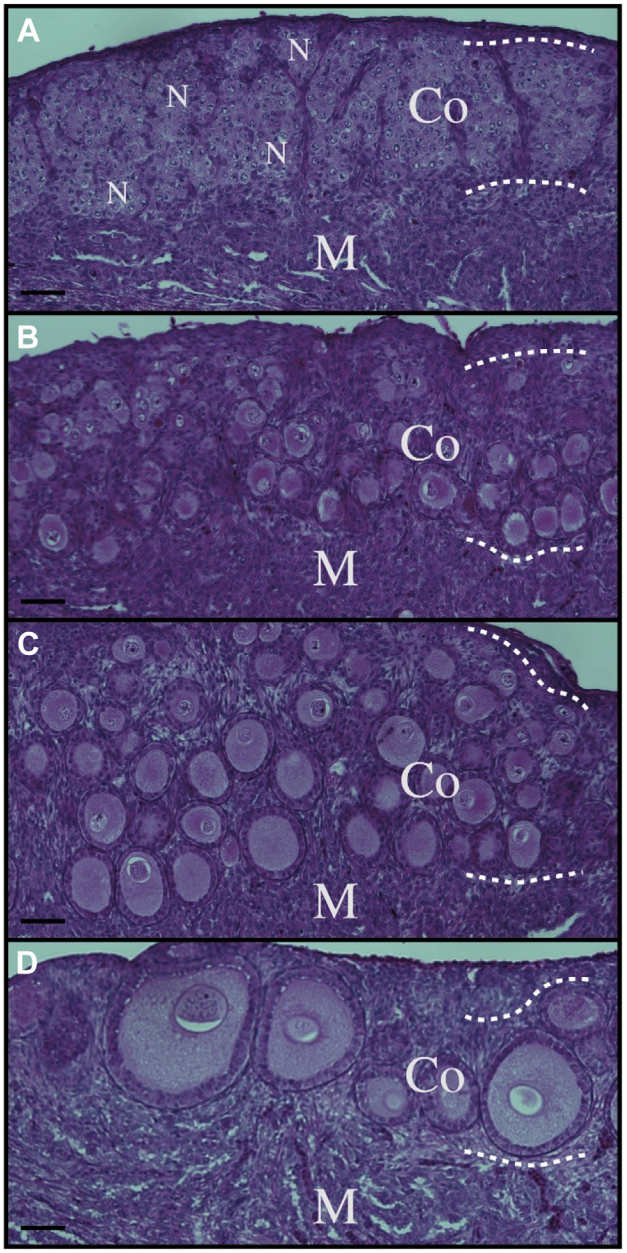

Figure 1. Histological appearance of the cortex **(Co)** and medulla **(M)** in the left ovary from white breasted turkey poults at 5 dph **(A)**, 9 dph **(B)**, 15 dph **(C)**, and 35 dph **(D)**. Individual germ cell nests **(N)** are defined based on their distance apart from each other, and the appearance of immature granulosa cells between them, which appear as purple lines, cutting through the cortex. The cortex is defined by a **dashed line**. Scale bars **(A–D)** 50 μm. Abbreviation: dph, days posthatch.

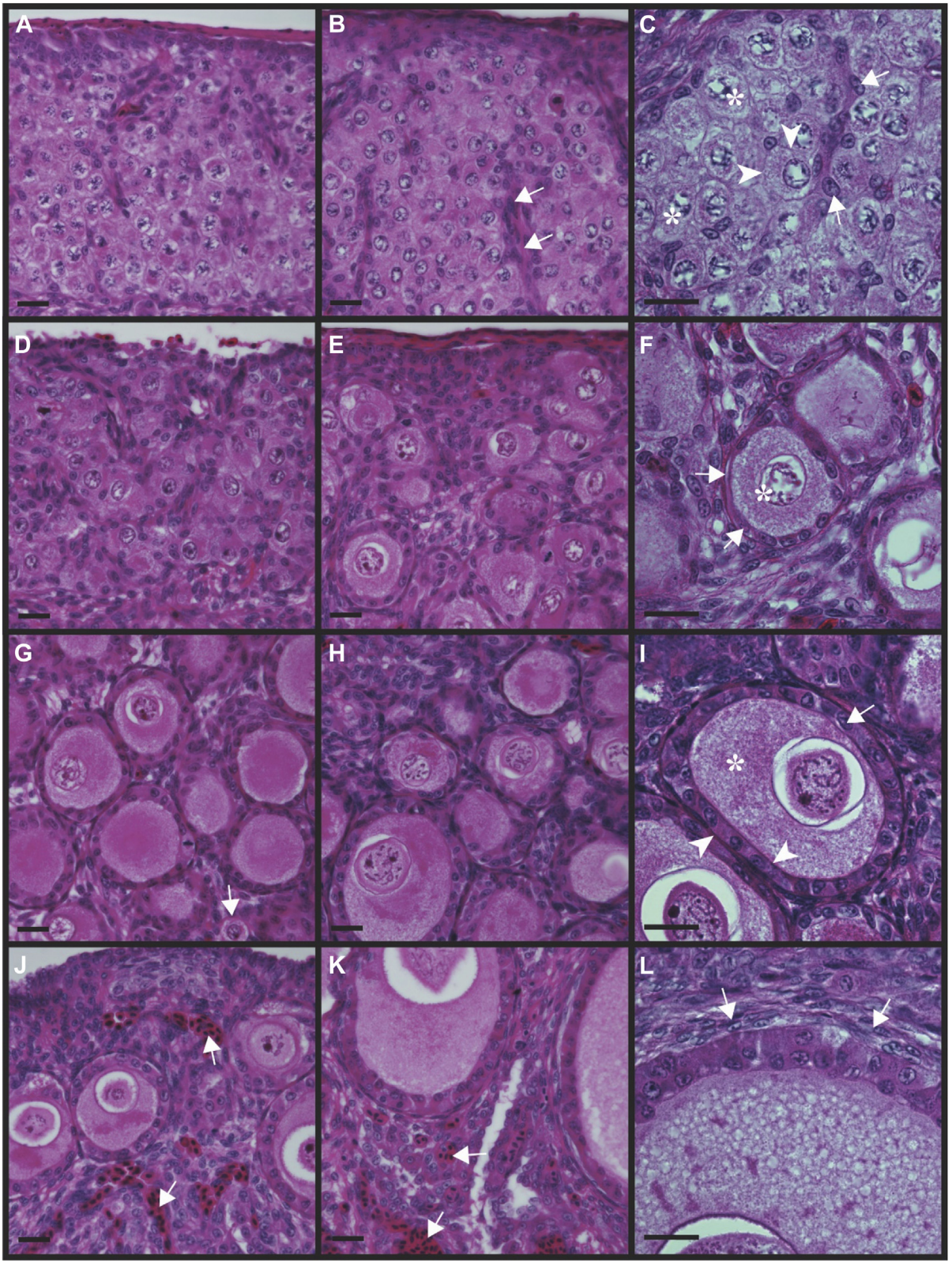

Figure 2. Histological appearance of the cortical tissue within the left ovary from white breasted turkey poults 1 to 35 dph. **(A)** 1 dph. **(B,C)** 5 dph, with prefollicular germ cells **(asterisks)** and immature granulosa cells **(arrows)** present within the cortex. For measuring the diameter of prefollicular germ cells, a clear germ cellular membrane **(arrow heads)** was visible. **(D)** 7 dph. **(E,F)** 9 dph, with primordial follicles **(asterisk)** being present, these early primordial follicles had granulosa cells **(arrows)** surrounding them that appeared squamous or flattened in shape. **(G)** 15 dph, prefollicle germ cells **(arrow)** were still present. **(H,I)** 21 dph, at this intermediate age primordial follicles **(asterisk)** had the characteristically normal cuboidal granulosa cells **(arrows).** For measuring the diameter of a primordial follicle and primary oocyte within, a clear external basement membrane and internal cellular membrane **(arrow heads)** were visible, respectively. **(J)** 28 dph, at this time point large blood vessels **(arrows)** appeared in the cortical region. **(K,L)** 35 dph, (K) blood vessels **(arrows)** became larger, and **(L)** immature theca cells **(arrows)** were present surrounding the largest of the primordial follicles. Scale bars (A–L) 20 μm. Abbreviation: dph, days posthatch.

Study 1

Prefollicular Germ Cell Diameter and Density Prefollicular germ cell diameter remained constant 13.2 ± 0.2 μm, ($P > 0.05$) during the first week posthatch (Figure 3A). Between 7 and 9 dph, the average diameter increased ($P \leq 0.05$) from 14.1 ± 0.2 to 17.6 ± 0.5 μm, respectively. No difference in cell diameter was observed between 9 and 11 dph ($P > 0.05$); however, a second increase ($P \leq 0.01$) was observed between day 9 and day 21 (22.4 ± 0.6 μm). Between 21 and 28 dph, a third increase ($P \leq 0.001$) in cell diameter occurred, reaching a final size of 26.4 ± 1.1 μm. Over the 4 wk, when prefollicular germ cells were present, the minimum size was 9.3 ± 0.8 μm with the maximum size being 40.9 ± 6.4 μm (Table 1).

While the density of prefollicular germ cells remained constant during the first 5 dph (648,587 ± 26,597/mm^3), a sharp decrease ($P \leq 0.001$) in density occurred at 7 dph (279,478 ± 52,239/mm^3), as shown in Figure 4A. The density decreased ($P \leq 0.01$) again on day 11 to 67,254 ± 20,165/mm^3. Prefollicular germ cells were still present by 28 dph within the cortex but were absent at 35 dph.

Primordial Follicle Diameter and Density Primordial follicles were first observed at 7 dph, with an average diameter of 37.5 ± 1.5 μm (Figure 3B). The diameter increased to 52.6 ± 2.2 μm ($P \leq 0.05$) on day 13 and again to 72.0 ± 2.6 μm ($P \leq 0.05$) by 28 dph. No further change ($P > 0.05$) in size was observed during the fifth week posthatch. Between 7 and 35 dph, the smallest primordial follicles were 30.0 ± 1.3 μm with the largest being 145.6 ± 29.1 μm (Table 1). Primary oocytes followed the same growth pattern as primordial follicles (Figure 3B), except the first increase ($P \leq 0.05$) in size for primary oocytes occurred between 7 and 11 dph.

As shown in Figure 4, the density patterns of prefollicular germ cells and primordial follicles were inversely proportional for the first 21 D. The density of primordial follicles increased ($P \leq 0.05$) from 438 ± 126/mm^3 at 7 dph to 3,316 ± 602/mm^3 by 11 dph and to 7,397 ± 777/mm^3 on day 15 ($P \leq 0.01$). There was no observed change in primordial follicle density between 15 and 21 dph. Primordial follicle density dropped ($P \leq 0.001$) by 28 dph to 3,726 ± 289/mm^3, with no significant change thereafter.

Percent Germ Cell and Follicle Volume During the first 3 time points posthatch, the total volume of germ cells in the cortex ranged from 65.9 ± 5.9% to 83.5 ± 6.5% and consisted exclusively of prefollicular germ cells (Table 2). When both prefollicular germ cells and primordial follicles were present, the total volume ranged from 42.0 ± 7.9% to 72.7 ± 4.6% of the cortex. No datum point ever exceed 100%.

Study 2

Cortex Volume, Germ Cell, and Follicle Counts The cortex volume steadily increased ($P \leq 0.05$) from 3.0 ± 0.6 mm^3 on day 5 to 27.9 ± 2.3 mm^3 by 35 dph (Table 3). While the cortex was growing the number of germ cells decreased ($P \leq 0.05$) from 1,057,402 ± 194,627 on day 5 to 60,768 ± 15,465 by 35 dph. During nest breakdown and follicle pool formation between 5 and 15 dph, a majority (~90%) of the prefollicular germ cells disappeared. It is unclear if prefollicular germ cells still present at 15 dph contributed to the final follicle pool, as there was no change in primordial follicle counts between 15 and 35 dph. By 35 dph, only primordial follicles were present, demonstrating that by this age the follicle pool was fully established.

DISCUSSION

Histological and stereological information on the turkey ovary during germ cell nest breakdown and formation of the primordial follicle pool is limited. Thus, the goal of the 2 studies here was to provide a thorough description of cellular depletion and reorganization events in the turkey ovary, during the first 5 wk posthatch. Our evaluation of poult ovarian morphology provides evidence that the cellular depletion event known as germ cell nest breakdown (1) initiates between 5 and 7 dph, (2) is largely completed by 15 dph, and is (3) fully concluded at 35 dph. At this last age, the primordial

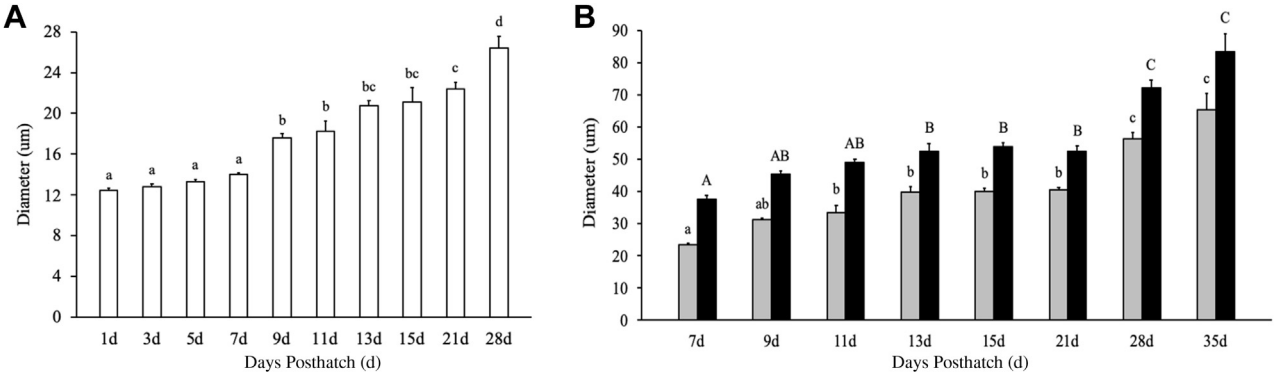

Figure 3. Diameter of **(A)** prefollicular germ cells and **(B)** primary oocytes (grey) and primordial follicles (black) in turkey ovarian tissue 1 to 35 days posthatch. (n = 4/age group, mean ± SEM). [a-d o,r A−C] Means with no common superscript within the same cell or follicle type differ significantly ($P \leq 0.05$).

Table 1. Diameters of smallest (min.) and largest (max.) prefollicular germ cells, primary oocytes, and primordial follicles from turkey ovaries 1–35 dph. (n = 4/age group, mean ± SD).

	Prefollicular germ cell		Primordial follicle			
			Primary oocyte		Follicle	
dph	Min. (μm)	Max. (μm)	Min. (μm)	Max. (μm)	Min. (μm)	Max. (μm)
1	9.3 ± 0.8[a]	16.5 ± 0.9[a]	-	-	-	-
3	9.2 ± 0.3[a]	16.3 ± 0.6[a]	-	-	-	-
5	9.9 ± 0.2[a,b]	17.1 ± 1.0[a]	-	-	-	-
7	10.4 ± 0.2[a,b]	21.2 ± 2.1[a]	18.2 ± 0.8[a]	27.8 ± 1.5[a]	30.0 ± 1.3[a]	45.7 ± 1.7[a]
9	11.6 ± 0.9[a,c]	29.8 ± 4.2[b]	22.9 ± 2.7[a,b]	42.3 ± 2.5[a,b]	33.2 ± 1.8[a]	61.4 ± 2.0[a,b]
11	12.0 ± 0.9[a,c]	29.5 ± 3.5[b]	25.8 ± 5.0[a,c]	44.2 ± 5.5[a,b]	35.5 ± 2.8[a,b]	66.7 ± 3.1[a,b]
13	13.2 ± 1.1[b,d]	32.3 ± 2.8[b]	29.7 ± 2.4[b,c]	52.3 ± 4.9[a,b]	39.1 ± 3.1[a,b]	73.8 ± 3.6[a,b]
15	13.1 ± 2.0[b,d]	32.0 ± 4.4[b]	30.5 ± 1.9[b,c]	58.6 ± 3.9[a,b]	39.9 ± 4.7[a,c]	81.0 ± 4.8[a,b]
21	14.2 ± 2.6[c,d]	33.2 ± 3.5[b,c]	28.8 ± 1.6[b,c]	67.9 ± 5.3[b]	36.0 ± 4.9[a,b]	86.7 ± 7.7[b]
28	16.5 ± 2.3[d]	40.9 ± 6.4[c]	33.9 ± 4.8[c,d]	107.6 ± 26.4[c]	47.1 ± 7.3[b,c]	132.2 ± 30.5[c]
35	-	-	41.1 ± 8.6[d]	120.5 ± 25.2[c]	52.9 ± 11.4[c]	145.6 ± 29.1[c]

[a-d]Means with no common superscript in the same column differ significantly ($P \leq 0.05$).
Abbreviation: dph, days posthatch.

follicle pool has been fully established, and no further follicles will be formed.

The overall histological change in the cortex of the turkey ovary was similar to that of the chicken and quail. One challenge experienced was properly identifying primordial follicles. Although the characteristics of primordial follicles are well defined (Johnson, 2015), in our study, intermediate forms were also identified. Be it an early primordial follicle, which often appeared with a squamous or flattened layer of granulosa cells or a late primordial follicle transitioning to a primary follicle, which appeared to be forming an external theca cell layer, although it was still well within the cortex. This highlights the trouble with defining key cellular characteristics, as this is a dynamic process with intermediate stages along the way.

To date, there has been only one other publication describing ovarian germ cell morphology in poults and that study evaluated ovaries for a 1 wk period posthatch (Liu et al., 2017), with a more limited microscopic evaluation. In general, the germ cell measurements at each time point (1, 3, 5, and 7 dph) from both studies were similar, although it was reported by Liu et al., 2017 that oogonia (described as prefollicular germ cells here) diameters increased significantly over time; whereas, no such significant growth was observed during the same period in our first study. Rather, the first increase in prefollicular germ cell size was observed between 7 and 9 dph. Prefollicular germ cells ranged in size from 9 to 41 μm in diameter, showing that prefollicular germ cells can grow before being incorporated into a well-defined primordial follicle. Because prefollicular germ cell maximum values approximated primary oocyte minimum values, it is possible that smaller prefollicular germ cells may not transition into becoming primary follicles. The size of oogonia and primary oocytes around the time of nest breakdown has been reported in older literature for mammalian species (Beaumont and Mandl, 1961; Baker, 1963; Ioannou, 1964; Black and Erickson, 1968) and the domestic chicken (Greenfield, 1966); germ cell size in relationship to age during nest breakdown has only been reported in the mouse (Pepling and Spradling, 2001). In the mouse, germ cells grow minimally (~3 μm) during nest breakdown and follicle formation. In contrast, a substantial increase in prefollicular germ cell and primary oocyte size occurs during the same process in the turkey. It is not known if other avian species follow the same growth dynamics.

Based on study 1, primordial follicles first appeared at 7 dph, which is similar to previous reports (Liu et al., 2017). In chickens, the maximum diameter of primordial follicles has been reported at 80 μm (Johnson, 2015). Here, we find that primordial follicles in the domestic turkey can range between 30 and 175 μm. The larger follicles appear to have primary follicle features, namely the start of an external theca cell layer, although they have not started to protrude out of the cortex, another defining feature of an avian primary follicle. There was no change in the average size (50–55 μm) of primordial follicles between 11 and 21 dph; however, there was an increase in the largest primordial follicle observed in each ovary during the same period. This seems to suggest that within the first 3 wk posthatch, some primordial follicles are growing or have been selected to grow while others have not. We know that this occurs in mature birds as ovaries possess follicles at all stages of development, even unrecruited primordial follicles (Apperson et al., 2017). To our knowledge, this is the first evidence to suggest that certain follicles in ovaries 1 to 3 wk posthatch are being selected to grow over others, potentially meaning that the follicle hierarchy is already being established.

The density of germ cells has been used to analyze cultured ovarian tissue (Beck et al., 2018); here, it was used to determine germ cell and follicle patterns. This method of evaluating germ cell numbers is more time efficient than determining total germ cell or follicle populations. In our first study, this allowed for the determination of important cellular time points before (5 dph), during (9 dph) and after the majority (15 dph) of germ cell nests had broken down. Germ cell nest breakdown was determined to occur between 5 and 7 dph, with the first significant decrease in prefollicular germ cell density and the first appearance of primordial follicles.

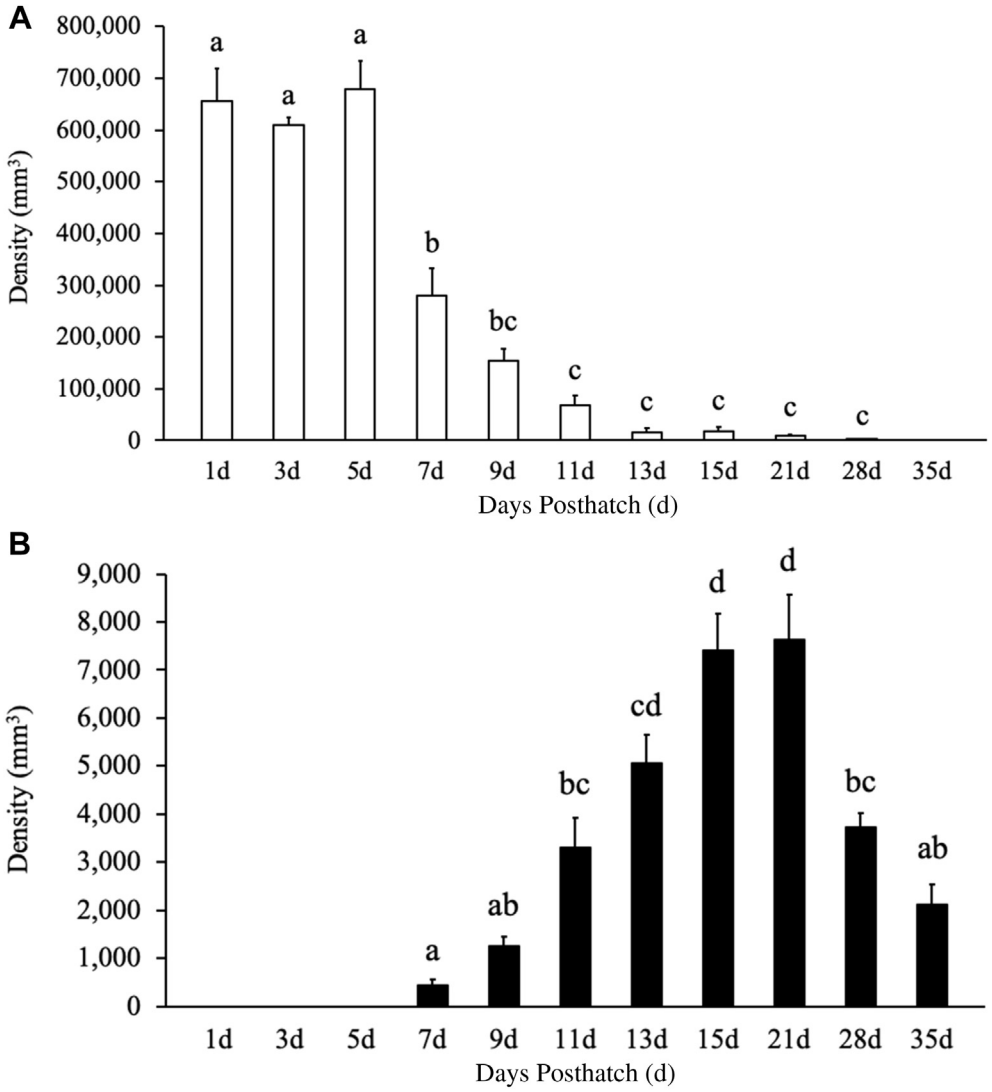

Figure 4. Density of **(A)** prefollicular germ cells and **(B)** primordial follicles in the cortex of the left ovary in poults 1 to 21 days posthatch (n = 4/age group, mean ± SEM). [a-d]Means with no common superscript differ significantly ($P \leq 0.05$).

Interestingly, this demonstrated that mass prefollicular germ cell loss initiated before the first substantial growth spurt of prefollicular germ cells, suggesting that the loss may be required to stimulate prefollicle germ cell growth.

A majority of germ cell nests were broken down by 15 dph as seen by the leveling off prefollicle germ cell density and primordial follicle density reaching their highest values. Primordial follicle densities dropped after 21 dph, not because of a decrease in primordial follicles, as primordial follicles numbers increased during this time period, but rather because of the growth of the cortex and primordial follicles within. Prefollicular germ cells were still present up until 28 dph demonstrating that by 35 dph the primordial follicle pool is fully established as prefollicular germ cells were no longer present. This occurs 1 wk later than reported in chickens in which the primordial follicle pool is established at 28 dph (Gonzalez-Moran, 2011). By identifying these key time points, we were then able to revisit these ages in our second study and determine the exact number of germ cells and follicles. Interestingly from the density data, we were also able to see that the decrease in prefollicular germ cell density followed an exponential trend, while the primordial follicle density increase was more linear between 7 and 15 dph. This suggested that a smaller percentage of prefollicular germ cells were incorporated into primordial follicles at the start of nest breakdown compared with the end.

There has been a substantial amount of controversy surrounding the accuracy of germ cell counts in the fetal and neonatal mouse ovary based on a 10-fold variation in germ cell numbers reported between groups (Tilly, 2003). It was then vital in our case to determine a method of validating our counting technique. We did this by using the relationship between size and density to determine the percent volume of germ cells and follicles within the cortex. Because our percentage of germ cell and follicle volumes never passed the maximum value of 100% or an unreasonably low number (<10%) based on the histological images, our counting technique was validated,

Table 2. Percent volume of prefollicular germ cells, primordial follicles, and the combined total within the cortex of the ovary in poults 1–21 dph. (mean ± SD).

dph	Prefollicular germ cells (% volume)	Primordial follicles (% volume)	Total germ cells and primordial follicles (% volume)
1	65.9 ± 5.9^{a,b}	-	65.9 ± 5.9^{a,c}
3	67.7 ± 2.8^{a}	-	67.7 ± 2.8^{a,c}
5	83.5 ± 6.5^{a}	-	83.5 ± 6.5^{c}
7	40.7 ± 8.9^{c}	1.3 ± 0.0^{a}	42.0 ± 7.9^{d}
9	43.2 ± 6.0^{b,c}	6.1 ± 0.8^{a,b}	49.2 ± 5.6^{a,d}
11	20.5 ± 5.3^{c,d}	21.3 ± 5.3^{b,c}	41.7 ± 1.0^{d}
13	7.0 ± 2.7^{d}	38.5 ± 5.0^{c}	45.5 ± 3.5^{a,d}
15	6.8 ± 2.9^{d}	60.4 ± 5.3^{d}	67.2 ± 2.7^{a,c}
21	5.3 ± 1.7^{d}	56.4 ± 3.1^{d}	61.6 ± 4.5^{a,d}
28	0.7 ± 0.3^{d}	72.0 ± 4.8^{d}	72.7 ± 4.6^{b,c}
35	-	59.3 ± 8.3^{d}	59.3 ± 8.3^{a,d}

$^{a\text{-}d}$Means with no common superscript in the same column differ significantly ($P \leq 0.05$).
Abbreviation: dph, days posthatch.

allowing us to proceed and determine germ cell and follicle populations.

From the 4 ages assessed in study 2 (5, 9, 15, and 35 dph) for germ cell and follicle counts, the total germ cell count was highest at 5 dph and decreased to 6% of this total by 35 dph. The total number of germ cells in our study exceeded that of other published reports in chickens (Hughes, 1963; Méndez-Herrera et al., 1998; Gonzalez-Moran, 2011), as ∼1,000,000 prefollicular germ cells were seen in the turkey at day of hatch, compared to 480,000 to 175,000 in the chicken. Moreover, ∼60,000 primordial follicles established the primordial follicle pool in the turkey compared with 50,000 in the chicken. Comparing germ cell counts between studies can be challenging for 2 reasons. First, methods for counting cells range widely from fixed histology to cell suspension, and nomenclatures, even when standardized can be subjectively interpreted by the observer. On the other hand, the stereological analysis of the cortex is based on a well-defined structure, subjective choice by the observer is eliminated, and the data are thus more reliable. The stereological analysis of the turkey ovarian cortex revealed that this structure is larger than that of the chickens, thus having the capacity to contain a higher number of germ cells. This, taken together with our count data, lends support that the turkey possess more germ cells than the chicken, at least during the period of the 2 turkey studies here.

We observed a 70% decrease (∼700,000) in prefollicular germ cells between 5 and 9 dph, with only a small (∼6,500) addition of primordial follicles. Whereas, between 9 and 15 dph when another roughly 150,000 prefollicular germ cells were lost, we saw roughly 60,000 primordial follicles added to the pool. This suggests that prefollicular germ cells lost at the start are not being added to the follicle pool compared with the ones which are lost toward the end of germ cell nest breakdown. This was also observed by our density data (exponential decay vs. linear growth), validating the density data as

Table 3. Number of prefollicular germ cells, primordial follicles, and the combined total within the cortex of ovaries from turkey poults at 5, 9, 15, and 35 dph (n = 4/age group).

dph	Bird (ID)	Cortex volume(mm^3)	Prefollicular germ cell (#)	Primordial follicle (#)	Total germ cell and follicle (#)
5	5A	4.2	1,485,590	-	1,485,590
	5B	2.6	1,106,305	-	1,106,305
	5C	1.7	540,459	-	540,459
	5D	3.3	1,097,255	-	1,097,255
		3.0 ± 0.6^{a}	**1,057,402 ± 194,627^{a}**	**-**	**1,057,402 ± 194,627^{a}**
9	9A	4.6	403,152	3,497	406,649
	9B	4.5	456,790	3,539	460,329
	9C	4.9	183,632	11,953	195,585
	9D	3.6	233,620	7,357	240,977
		4.6 ± 0.3^{a,b}	**319,299 ± 65,626^{b}**	**6,587 ± 2,005^{a}**	**325,885 ± 63,761^{b}**
15	15A	8.4	113,953	54,476	168,429
	15B	11.7	161,549	97,671	259,220
	15C	9.9	53,643	75,245	128,888
	15D	7.1	83,513	38,261	121,774
		9.3 ± 1.0^{b}	**103,165 ± 23,028^{b}**	**66,413 ± 12,878^{b}**	**169,578 ± 31,594^{b}**
35	35A	25.0	-	34,691	34,691
	35B	23.2	-	39,291	39,291
	35C	30.6	-	101,837	101,837
	35D	32.9	-	67,253	67,253
		27.9 ± 2.3^{c}	**-**	**60,768 ± 15,465^{b}**	**60,768 ± 15,465^{b}**

$^{a\text{-}c}$Means with no common superscript in the same column differ significantly ($P \leq 0.05$).
Abbreviation: dph, days posthatch.
Bold indicates mean ± SEM of the 4 datum points above.

an accurate method for analyzing population dynamics. The overall loss of germ cells during the observed 5 wk period was 94%. With this high percent loss of germ cells occurring in the domestic turkey and roughly 70 to 90% loss being reported in the domestic chicken (Hughes, 1963; Gonzalez-Moran, 2011), it appears that chickens and turkeys lose more germ cells during this period than mammals, which lose only an estimated two-thirds of germ cells during germ cell nest breakdown (Baker, 1972; Pepling and Spradling, 2001).

The reason for this potential higher rate of germ cell loss in turkeys compared with mammals is unclear. Explanations as to why germ cells are lost during nest breakdown mainly revolve around 3 theories: (1) chromosome dysfunction/mispairing or genetic defects; (2) limited trophic factors; and (3) donation of cytoplasmic components (McClellan et al., 2003; Felici, 2005; Tingen et al., 2009). Theory one is that a number of defects within a germ cell (dysfunctional telomeres, chromosome mispairing, mutations in genes) could result in elimination of that specific germ cell from the pool, thus providing a quality control step (Burgoyne and Baker, 1985; Barlow et al., 1998; Yoshida et al., 1998; Hemann et al., 2001). Because turkeys have a higher number of macrochromosomes and microchromosomes (Tegelstrom and Ryttman, 1981; Rodionov, 1996) than mammals (Painter, 1925), it is possible that there are higher numbers of mispaired chromosomes during meiosis which might be a factor contributing to the increased level of germ cell loss. The second theory is based on the observations that treatment of cultured female germ cells with specific growth factors prevented cell death (Godin et al., 1991; Matsui et al., 1991; Koshimizu et al., 1995; Morita and Tilly, 1999; Tilly, 2001). If these growth factors were limited during germ cell nest breakdown, then only germ cells that received enough or have greater sensitivity to growth factors would survive. To date, this has not been evaluated in birds. The third theory is based on 2 observations in mice where an increase in the number of mitochondria in an oocyte lowered its chances of being apoptotic (Perez et al., 2000) and that mitochondria have been found in the ring canals joining 2 germ cells together in the same nest (Pepling and Spradling, 2001). If more mitochondria mean primary oocytes have a greater chance of surviving, then potentially the number of primary oocytes acting as "nurse cells" affects the survival rate of the central primary oocyte. Both of these last theories focus on the growth of the germ cell, either with trophic factors or by providing 1 germ cell with a greater potential to develop. During nest breakdown in mice, (e.g., 19.5–25.6 D postconception), the average diameter of germ cells only increased by 20% (Pepling and Spradling, 2001). Conversely, our results show that during the same cellular depletion period in the turkey (e.g., 5–11 dph), the average diameter of germ cells increased 40 to 150% depending on whether the germ cells had been incorporated into primordial follicles or not. As oogenesis in mammals is known to require a significant amount of energy (Arhin et al., 2018), it seems likely that the extra growth in the turkey germ cell would require even more energy and cellular components, either in the form of higher levels of trophic factors and/or an increase in the number of germ cells acting like nurse cells to supply mitochondria and cellular components to the central primary oocyte. Either of these scenarios could result in greater competition for avian germ cells and explain why turkeys exhibit a larger depletion of germ cells during nest breakdown.

In summary, we have characterized the fundamental cellular changes within the ovary of the domestic turkey posthatch. It is clear that turkeys possess more germ cells, posthatch, than the domestic chicken. However, they exhibit a comparable percent decrease in germ cell numbers during nest breakdown. Although this percentage decrease has only been reported for 2 avian species, it is clear that early ovarian development in birds differs from that of mammals. Further studies will determine how and if these early ovarian cellular changes have any effect on poultry reproductive performance.

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