In ovo culturing of turkey (*Meleagris gallopavo*) ovarian tissue to assess graft viability and maturation of prefollicular germ cells and follicles

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ABSTRACT Biobanking of turkey ovarian tissue appears to be the most cost-effective method for the long-term preservation of female genetics. However, to ensure the successful transplantation of biobanked ovarian tissue for breed or line revival, the transplantation and development of fresh ovarian tissue must be evaluated. To assess transplantability, ovaries from poults 1 to 15 days posthatch (**dph**) were cultured *in ovo* in chicken eggs for 6 d and compared with the equivalent fresh tissue. The viability of cultured ovarian tissue was evaluated visually, whereas the level of late-stage apoptosis was measured via the TUNEL assay. In addition, the diameter and density of prefollicular germ cells and follicles (primordial and primary) were measured to assess maturation. Results showed that all cultured grafts (74/74), on surviving chicken chorioallantoic membrane, were viable with low levels (0.8 ± 0.1%) of late-stage apoptosis. The diameter of prefollicular germ cells in cultured ovaries from poults at 5 and 7 dph were larger ($P < 0.002$) than that of their preculture counterparts but were not able to reach their *in vivo* size. No significant follicular growth was observed in ovaries cultured *in ovo*; however, prefollicular germ cell density was over 4-fold greater in ovaries cultured from 7 dph poults (81,030 ± 17,611/mm^3) than in their *in vivo* counterpart (16,463 ± 6,805/mm^3). Interestingly, cultured ovaries from all other ages displayed equal or lower ($P \leq 0.05$) prefollicular germ cell densities than their *in vivo* counterparts. Cultured ovaries from poults at 5 and 7 dph also exhibited an increase ($P \leq 0.05$) in follicle density compared with their preculture counterparts; whereas, cultured ovaries from 15 dph poults had decreased densities ($P < 0.001$) compared with their preculture counterparts. This study demonstrated that, although age of ovarian tissue cultured *in ovo* did not affect the overall viability, 7 dph ovaries appeared to have a better cellular morphology after culturing *in ovo* than other ages. In addition, we also demonstrated for the first time that avian follicles can form during tissue culturing *in ovo*.

Key words: turkey, ovary, *in ovo* culture, prefollicular germ cell, follicle

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INTRODUCTION

Biobanking of poultry ovarian tissue and semen as a means of preserving the entire genome is fast becoming a reality. Cryopreservation of ovarian tissue is required along with semen due to the fact that in birds, the female is the heterogametic sex (*WZ*) and provides the mitochondria DNA. Without the ovarian tissue being incorporated into poultry cryopreservation strategies, important genetic material would be lost. In the case of the domestic turkey, semen cryopreservation techniques have improved (Iaffaldano et al., 2016), although for many breeds, fertility rates from cryopreserved semen may still be too low to be relied on for guaranteed reconstitution (Long et al., 2014). On the other hand, cryopreserved turkey ovarian tissue has not been successfully transplanted into recipient poults and therefore ovarian maturation and production of donor-derived progeny have not been possible. Developing a successful cryopreservation and transplantation protocol for turkey ovarian tissue is therefore necessary.

As a first step, before cryopreserved tissue can be used, it is important to establish and validate a protocol for

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the transplantation of fresh ovarian tissue. Successful fresh tissue transplantations have been reported in other species such as chicken, quail, and duck, with success measured either by donor-derived progeny or via genotyping of the transplants (Song and Silversides, 2007, 2008a, b; Song et al., 2011; Liptoi et al., 2020). In the case of donor-derived progeny, the highest success rates for chicken, quail, and duck have been 40% (4/10), 63% (5/8), and 13% (1/8), respectively. Similarly, when fresh chicken transplants were evaluated by genotyping transplants from recipient birds at 8, 11, and 30 wk of age, 52% (10/19) of the hens still possessed transplants (Liptoi et al., 2020). Interestingly, quail had the highest reported success rate suggesting species-specific differences; however, transplantations were performed using ovaries collected at 7 days posthatch (**dph**) in quail, and 1 dph in chickens and ducks. This raises the possibility that a more mature tissue is required for successful transplantations and species difference may have been confounded with transplant age.

A number of reasons may be responsible for the reported failures of ovarian transplantation in poultry, which include poor graft attachment rate, insufficient revascularisation leading to ischemia, reperfusion injury, and graft rejection by the recipient's adaptive immune system (Song and Silversides, 2007; 2008b). Even when the graft develops successfully, the transplanted ovary may be incorrectly positioned within the abdominal cavity or the oviduct could have been inadvertently damaged during the surgical procedure, both would result in unwanted internal ovulations (Liptoi et al., 2020).

To effectively assess the impact of donor age on ovarian transplantation success rates in the domestic turkey, we opted to utilize a chorioallantoic membrane (**CAM**) *in ovo* culture system. This requires attachment and revascularization of the transplant to the CAM of an early-stage chicken embryo. The benefit being that this culture system avoids the possible immune rejection, which could occur if the tissue was transplanted into a recipient poult because the chicken embryo does not reach immunocompetency until the 14th d of embryonic development (Tizard, 2009). This system also guarantees immobilization of the graft because the graft remains stationary with respect to the CAM. However, this system does mimic certain conditions that the transplanted ovarian tissue would experience in a recipient poult, with 1 to 3 d of ischemia followed by reperfusion after revascularization to the CAM blood vessels (Beck et al., 2018). The successful culturing *in ovo* of fresh and cryopreserved ovarian tissue has been demonstrated in several other species including cattle (Cushman et al., 2002; Gigli et al., 2005; Beck et al., 2018; 2020), mice (Gigli et al., 2005), sheep (Qureshi et al., 2008; Vatanparast et al., 2018), humans (Martinez-Madrid et al., 2009; Isachenko et al., 2012; 2013a, b), cats (Vilela et al., 2016), chickens (Yuan et al., 2009), and quail (Liu et al., 2012). In the quail study, the focus was on cryopreserved tissue, although a fresh tissue control (7 dph) was also cultured for 5 to 6 d. Only 53% (9/17) of these fresh quail grafts survived with good or partial vascularization, whereas the others failed due to poor vascularization and necrosis (Liu et al., 2012). Interestingly, these results from the *in ovo* culturing of quail ovaries closely mirrors the success rate (63%) of ovarian transplants in live quail (Song and Silversides, 2008b), suggesting that the *in ovo* culture system may be appropriate to predict the success rate of grafts transplanted into recipient birds.

Using an *in ovo* CAM culture system, turkey ovarian tissues ranging from 1 to 15 dph were cultured for 6 d and were then visually graded for vascularization, processed for histological analyses, and evaluated for late-stage apoptosis (cell death) using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The aim was to determine if tissue viability, prefollicular germ cell, and follicle maturation and development within transplanted turkey ovarian tissue was affected by the age of the donor.

MATERIALS AND METHODS

Animals and Sampling

Fertilized White Leghorn chicken eggs obtained from the University of Guelph poultry research station (Guelph, Ontario, Canada) were used for the *in ovo* tissue culturing protocol. After tissue culturing, all White Leghorn embryos were euthanized at 15 d of incubation by decapitation. Day-old female poults were supplied by Hybrid Turkeys (Kitchener, Ontario, Canada) from a female parent-stock line. Poults were housed at the University of Guelph's Animal Care Facility in group floor pens and given *ad libitum* food and water. Photoperiod was set at 23 h during the first dph followed by a stepwise 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 21 dph, with the age defined as dph ±6 h based on the hatch window. The use of animals and all procedures were reviewed and approved by the Institutional Animal Care Committee and adhered to the principles described by the Canadian Council on Animal Care.

After euthanasia, the poult's abdomen was opened and the left ovary removed and placed into a petri dish containing holding media (PBS with 20% fetal bovine serum) at room temperature. Each ovary was then cut into 2 × 2 mm segments (3 segments per ovary) under a dissecting microscope. After dissection, ovaries from poults (n = 4) at 1, 3, 5, 7, 11, and 15 dph were immediately fixed and histologically processed as fresh controls to represent ovarian morphology at the start of each time point (preculture ovaries; Table 1). A second set of ovaries from poults at 1, 3, 5, 7, 11, and 15 dph (n = 4 or 5/age) were instead cultured *in ovo* for 6 d (cultured ovaries; Table 1). Each ovarian segment was *in ovo* cultured separately, making them technical replicates. If the chicken embryo died during culturing *in ovo*, the experimental replicate was removed from the study. If 2 or more experimental replicates were removed for

Table 1. During this study, ovaries were classified as preculture, cultured, and *in vivo*.

Preculture (dph)	Cultured (dph)	*In vivo* (dph)
1	1 + *in ovo*	7
3	3 + *in ovo*	9
5	5 + *in ovo*	11
7	7 + *in ovo*	13
11	11 + *in ovo*	17
15	15 + *in ovo*	21

Preculture refers to fresh ovaries which have yet to be cultured, whereas cultured ovaries refer to ovaries which have been cultured *in ovo* for 6 d. The age at which *in ovo* culturing began is listed first followed by "+ *in ovo*". *In vivo* fresh ovaries represent normal ovarian development after 6 d, which is the same length of time the *in ovo* grafts were cultured. For each age within the 3 classifications, 4 poults (n = 4/age) each provided 3 ovarian segments as technical replicates.

Abbreviation: dph, days posthatch.

one particular ovary, the ovary was removed from apoptosis and cellular morphology analysis, although the remaining graft was measured for size and visually assessed for vascularization. This occurred for one ovary at the 3 and 7 dph ages; in addition, one ovary from the 11 and 15 dph time points was also randomly removed from the apoptosis and cellular morphology analysis, so as to maintain an equal sample size (n = 4) for each age group. A final set of ovaries from different poults at 7, 9, 11, 13, 17, and 21 dph (n = 4 per age class) were also immediately fixed for histological processing to represent the normal cellular development of ovaries during this 6 d period (*in vivo* ovaries; Table 1). Bursa of Fabricius from the 11 dph poults were also collected, fixed, and processed as a source of known apoptotic tissue.

In Ovo Tissue Culturing

Culturing *in ovo* of turkey ovarian tissue was performed in a similar manner to previous studies (Qureshi et al., 2008; Martinez-Madrid et al., 2009; Liu et al., 2012; Isachenko et al., 2013b; Beck et al., 2020). Initially, 112 fertilized White Leghorn eggs were rinsed with water, wiped with 70% ethanol, and then incubated in an egg incubator with a rotating tray (1588 Genesis Hova-Bator with 1611 Automatic Egg Turner; GQF Manufacturing Company, Savannah, GA) for 3 d. The eggs were incubated at a constant 37.5°C and a relative humidity of 71 to 74%. On day 3 of incubation, eggs were moved to a stationary plastic egg tray, placed in a horizontal position and returned to the incubator for a further 2 h. Windowing was performed one egg at a time, in a biosafety cabinet. First, a small hole was created at the apex of the large end of the egg. Albumin (2 mL) was then aspirated through the hole using an 18-gauge needle without damaging the yolk sac. Next, the egg was positioned horizontally and a window (1 × 2 cm) was made in the eggshell using a rotary tool (DREMEL 100; Dremel, Racine, WI) with cutoff wheel attachment (No. 420; Dremel, Racine, WI). A piece of clear plastic tape was used to cover the windowed area, and the egg was placed back in the incubator on the stationary tray, window side up. For the next 6 d, embryos were observed daily for heartbeat and eggs containing dead embryos were removed. On day 9, grafting of ovarian tissue was performed in a biosafety cabinet. First, the tape covering the window was removed and the CAM was manually traumatized by laying a sterilized (dipped in acetone) strip of filter paper (9–12 mm^2) onto an area adjacent to major blood vessels. The filter paper was removed gently to minimize damage and the ovarian tissue segments were then placed on the area of traumatized CAM with the medulla side lying directly on the CAM (Figure 1B). The importance of ensuring the blood vessel–rich medulla is in contact with the CAM has previously been demonstrated, as ovarian grafts with both medulla and cortex have a higher success rate of grafting than grafts with only cortical tissue (Isachenko et al., 2012). The window was then resealed with tape, and the egg returned to the incubator. The entire grafting procedure was completed within 5 min or less. At this stage, the 5 control embryos did not receive grafts and were left in the incubator. On day 15 of incubation (6 d after ovarian grafting), the eggs were removed from the incubator, and the entire CAM surrounding the graft was exposed by removing the tape and more of the egg shell (Figure 1C). The grafts and surrounding CAM were photographed and excised using a dissecting microscope (Leica EZ4 W; Optic-Tech Scientific Inc., Pickering, Ontario, Canada), and both graft and surrounding CAM were removed and fixed for histological examination (Figure 1E). All chemicals and supplies were purchased through Fisher Scientific (Hampton, NH) unless otherwise stated.

Measurement and Grading of Cultured Grafts

The area of the grafts was determined by measuring the width and length from the captured images. Grafts were then visually assessed as previously reported in other *in ovo* culture studies using human (Martinez-Madrid et al., 2009), sheep (Qureshi et al., 2008), and quail (Liu et al., 2012) ovarian tissue. In our study, well-vascularized, defined as blood vessels can be seen over the entire graft, and partially vascularized, defined as less than 50% of the graft appearing to possess blood vessels, were combined into one group referred to as well/partly vascularized grafts (Figure 1F). Our rationale for combining these groups is that vascularization is by nature a three-dimensional property of the tissue, whereas visual assessments are based on 2 dimensions. Consequently, tissue assessed as partially vascularized could be more vascularized than well-vascularized tissue if it had more blood vessels closer to the center of the graft, and the well-vascularized tissue only possessed blood vessels within its peripheral edges. The other 2 types of grafts identified here were poorly vascularized grafts, defined as being attached to the CAM but appearing white, with no blood vessels, and dead grafts which appeared yellow or brown.

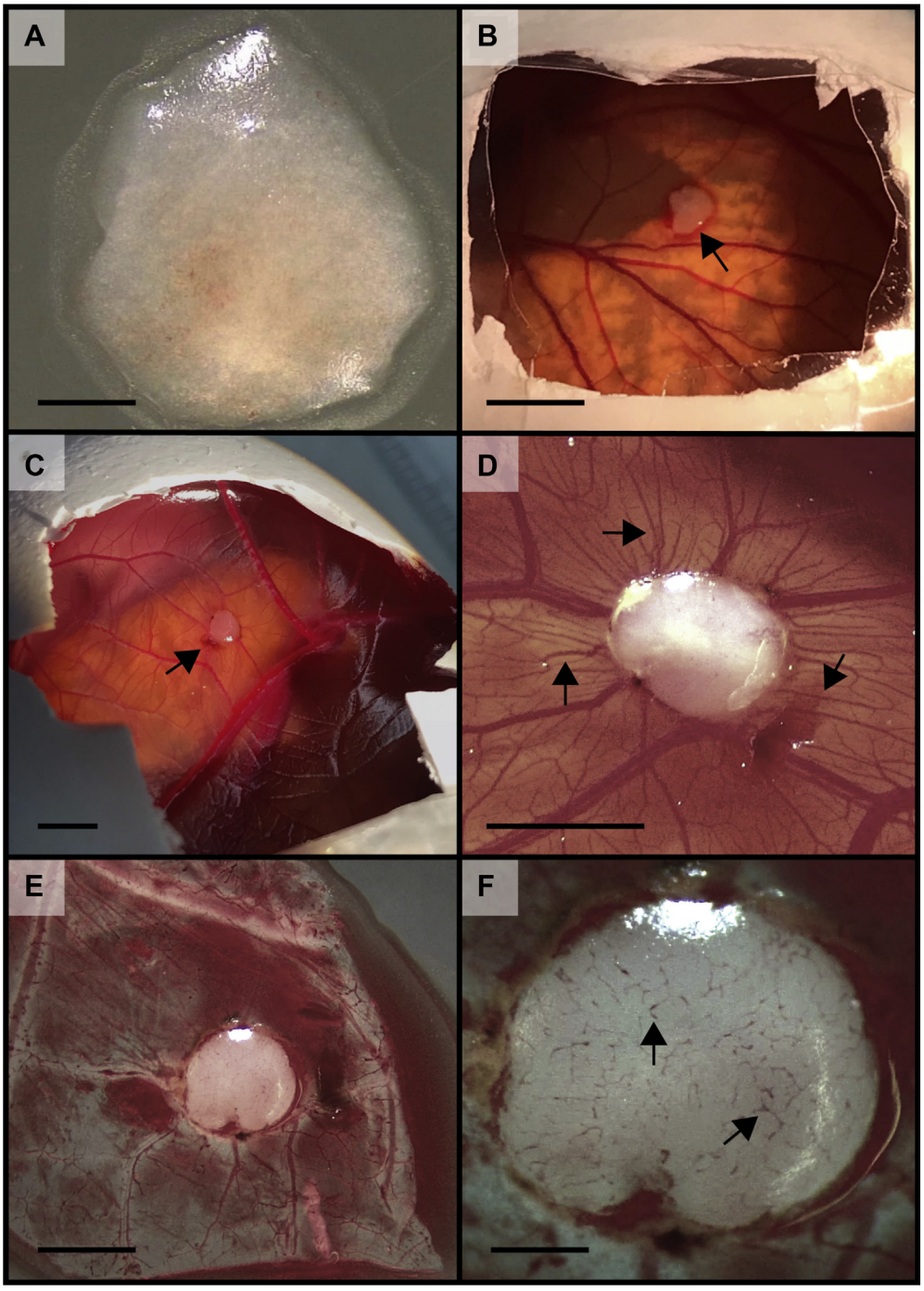

Figure 1. *In ovo* culturing of turkey ovarian tissue. (A) Preculture fresh ovarian tissue with no obvious signs of blood vessels. (B) Preculture ovarian tissue (arrow) placed onto the agitated CAM. (C) After 6 d of culturing the graft (arrow) appeared pink, and the CAM shiny. (D) Magnified view (5×) showing blood vessels in the CAM (arrows) being positioned in a pinwheel formation around the grafts. (E) Excised CAM containing a graft laid out flat on a petri dish. (F) Magnified view (4×) as an example of well/partly vascularized graft with large novel blood vessels (arrows). Scale bars (A, F) 0.5 mm, (B, C) 4.0 mm, (D, E) 2.0 mm. Abbreviation: CAM, chorioallantoic membrane.

Tissue Processing and Imaging

All tissues were fixed using Bouin's fixative, for 1 h at room temperature, and then for an additional 23 h at 4 C (Liu et al., 2017; Hall et al., 2020). For the cultured grafts, fixation was performed within a small petri dish, with the CAM spread out on the petri dish surface, so as to allow the surrounding CAM to be fixed in a flattened position. After fixation, all tissues were washed with PBS and transferred into tissue cassettes. For the cultured grafts, a small sponge was also placed within the cassette to keep the surrounding CAM in a flattened position. The cassettes were then stored in 70% ethanol at 4°C until further processing. All tissues were dehydrated, cleared with xylene, embedded with paraffin wax, and immobilized in paraffin blocks as previously reported for dissected ovaries (Hall et al., 2020) with the exception of the positioning of the cultured graft within

the block. Here, grafts were positioned with the CAM perpendicular to the cutting plane, allowing the sectioning of each graft to occur in a similar orientation. All tissues were sectioned at a thickness of 5 μm using a Finesse ME microtome (Thermo Shandon, Cheshire, UK). For each block, 4 serial sections were collected per slide with 100 μm (20 sections) discarded between slides. For preculture and *in vivo* ovaries, 8 to 10 slides were collected per block; whereas for the cultured ovarian grafts, slides were collected throughout the entire graft (10–17 slides per block). For the bursa of Fabricius blocks, 3 sections were randomly collected throughout each of the samples.

For all ovaries (preculture and *in vivo*) and cultured grafts, every other slide was stained with hematoxylin and eosin (H&E). For the TUNEL assay, one of the sections on a randomly selected slide was analyzed per segment or per graft for preculture, *in vivo*, or cultured ovaries (3 sections per ovary), whereas all the bursa of Fabricius sections were evaluated for apoptosis. The *in situ* Apoptosis Detection Kit (ab206386, Abcam Inc., Toronto, Ontario, Canada) was used according to Gao et al. (2017); however, hematoxylin was used instead of methyl green for counterstaining. Slides from bursa of Fabricius were used as a positive control as this tissue is known to have naturally high levels of apoptosis. All slides (H&E and TUNEL) were examined under a Leica DM 5000B light microscope (Leica, Wetzlar, Germany) equipped with a B-Series LED light source (ScopeLED, Richmond, CA) for sequential RGB imaging. For the H&E slides, the first section on each slide was imaged using a Hamamatsu Orca-Flash 4 camera (Hamamatsu Photonics, Hamamatsu City, Japan). Images were captured (mag. 40×) over the entire ovary section; these images were then subsequently stitched together to give a large, high-resolution image, using Volocity ver. 6.3.1 (Quorum Technologies, Guelph, ON, Canada). For the TUNEL sections, 3 random images of the cortex area of the segment or graft were captured at mag. 40× from each section. In the case of the bursa of Fabricius, the 3 images were taken within bursa follicles for each sample. This meant that for late-stage apoptosis analysis, a total of 9 images were used per ovary, and per bursa of Fabricius.

Diameter and Densities of Prefollicular Germ Cells and Follicles

Diameter and density measurements were calculated using the method reported by Hall et al. (2020), using the high-resolution images, with the exception that the 3 types of follicles (primordial, growing and primary) defined by Guo et al. (2019a, b) and Zhao et al. (2017) were combined into a single classification and referred to as "follicles". With respect to diameter measurements, 40 prefollicular germ cells, and 10 follicles were sampled per segment or graft, giving a total of 120 prefollicular germ cells and 30 follicles per ovary. For prefollicular germ cell and follicle densities, 2 high-resolution images were randomly selected and used per segment or graft, meaning 6 images were used per ovary. The values obtained from each ovary (preculture, cultured, and *in vivo*) not individual segments or grafts were then statistically compared.

Percentage of Late-Stage Apoptotic Cells

To determine the percentage of late-stage apoptosis within each of the randomly captured images, 200 nuclei were analyzed per image. Nuclei that appeared dark brown (Figure 2B) and condensed were considered apoptotic (TUNEL positive), whereas nuclei that appeared blue or light blue (Figure 2A) were considered not going through late-stage apoptosis (TUNEL negative). The number of TUNEL positive cells within all captured images was summed and divided by the total number analyzed and converted into percentages.

Statistical Analysis

Statistical analyses were performed using SPSS 25.0 for Mac (SPSS Inc., Chicago, IL). For graft size, percentage of TUNEL-positive cells, and prefollicular germ cell and follicle diameter and density, the data were presented as means ± SEM. Normality and equal variance of data were evaluated by residual plots and Levene's tests, respectively. For graft size, a one-way ANOVA was used to analyze the variance based on age of the tissue at the start of culture. If age had a significant ($P \leq 0.05$) effect, then a post hoc (Tukey) test was run to determine which ages differed. For TUNEL percentage, diameter, and density analysis, a two-way ANOVA was used to analyze the effects of ovary type (preculture, cultured, and *in vivo*) and age. If the interaction (type vs. age) was shown to be significant, a "simple effects" test was run to determine differences within ovary types at certain ages.

RESULTS AND DISCUSSION

The number of studies across several poultry species and breeds demonstrating that ovarian transplants can graft, mature, and eventually produce donor-derived progeny is encouraging (Song and Silversides, 2007, 2008a, b; Song et al., 2011; Liu et al., 2015; Liptoi et al., 2020). However, the mechanisms and factors that influence transplant success rates are for the most part still poorly understood. Knowledge of this information is crucial if this procedure is to be routinely used to conserve female poultry germplasm through biobanking. To that end, the present study investigated the potential impact of donor age on graft success rates. This was accomplished by culturing turkey ovarian tissue at varying ages, using the *in ovo* (CAM) culture system.

Chicken Embryo Survival Rates

The overall chicken embryo survival rate was 71% (79/112), which was lower than previously reported for

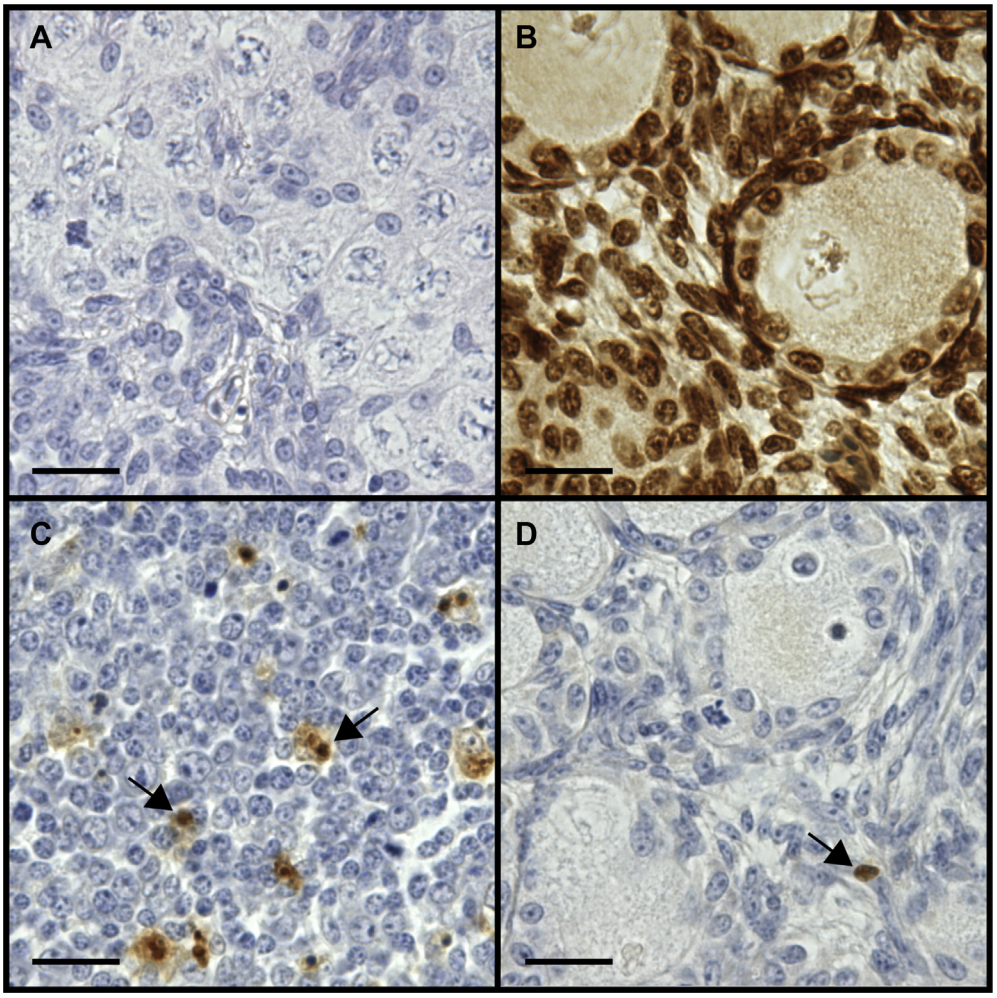

Figure 2. Late-stage apoptosis demonstrated via the TUNEL assay in turkey ovarian tissue (A, B, D) and bursa of Fabricius (C). (A) Negative control, with water replacing terminal deoxynucleotidyl transferase. (B) Positive control, with ovarian tissue treated with DNase1 before the quenching step. (C) Bursa of Fabricius (known apoptotic tissue) saw an increase in TUNEL-positive cells (arrows) which can be distinguished compared with normal nuclei appearing blue. (D) Cultured grafts contain a small number of apoptotic cells (arrow) as most cell nuclei appear blue. Scale bars (A–D) 20 μm.

CAM culture systems (Martinez-Madrid et al., 2009; Isachenko et al., 2013a; Beck et al., 2018, 2020). By day 3 of incubation, 96 of the 112 (86%) eggs were fertile and had survived the windowing procedure. Of these 96 embryos, 89 (93%) survived to the ninth day of incubation (after windowing), a higher survival rate compared with previously published values (Beck et al., 2018). The survival rate between the 9th and 15th d of incubation (after CAM traumatization) was slightly lower (89%; 79/89) than comparable studies (Martinez-Madrid et al., 2009; Isachenko et al., 2013a; Beck et al., 2018, 2020). Of the 79 embryos that survived till the 15th d of incubation, 74 contained successfully cultured turkey ovarian tissue segments, the other 5 were the controls which lacked grafts. The higher embryonic mortality rate between the 9th and 15th d of incubation could have been caused by the duration of the CAM procedure, since it was not possible to perform this procedure under the recommended 3-min threshold, which has been suggested for CAM traumatization and graft placement (Qureshi et al., 2008). Furthermore, it has been shown that when traumatization of the CAM and transplantation exceeded 7 min, the embryonic survival rate dropped to 20% (Qureshi et al., 2008). Given these metrics, it is possible that the 5-min duration of our procedure adversely impacted embryo survival. Another possible factor is the higher humidity in our incubators (71–74%) compared with the standard 60% reported in previous studies (Qureshi et al., 2008; Martinez-Madrid et al., 2009; Liu et al., 2012; Vatanparast et al., 2018), as high humidity has been shown to decrease embryo survival rates (Christensen et al., 2006). Notwithstanding, a sufficient number of embryos survived, and all surviving embryos facilitated the successful vascularization of the turkey ovarian grafts.

Visual Assessment of Turkey Ovarian Grafts

The success of human, sheep, and quail ovarian tissue to vascularize to the CAM of a chicken embryo has been defined by the grafts appearance and its level of attachment to the CAM (Qureshi et al., 2008; Martinez-Madrid et al., 2009; Liu et al., 2012; Vatanparast

et al., 2018). Here, a similar grading scheme was used to evaluate the *in ovo* cultured turkey ovarian tissue. The turkey ovarian tissue did not show any easily visible blood vessels before culture (Figure 1A). After culturing *in ovo*, the blood vessels within the CAM were organized in a pinwheel formation around the grafts (Figure 1D), while the surrounding CAM appeared glossy/shiny and lacked any keratinization (Figure 1C). All grafts on viable chicken embryo CAM appeared well/partly vascularized, regardless of age (Table 2). The success rate here (100%) is higher than in any of previously mentioned studies. Three possible reasons for the success rate here are the size of the ovarian grafts, the developmental stage of the CAM, and the humidity level. When large ovarian pieces (1 × 2 × 8 mm and 1 × 1 × 5 mm) from sheep were grafted onto CAM 3 to 4 d earlier than in our study, fewer (44–46%) ovarian grafts exhibited revascularization (Qureshi et al., 2008; Vatanparast et al., 2018). By contrast, when smaller pieces (1 mm^3, human) were grafted onto CAM 1 d later than in our study, the revascularization was much higher (83%), this being the closest rate of revascularization compared with our study (Martinez-Madrid et al., 2009).

As *in ovo* culturing of quail ovarian tissue using parameters similar to ours (ovarian pieces measuring 2.5 × 2.5 mm; CAM at day 8 of incubation) resulted in only 53% survival rate (Liu et al., 2012), the size of the graft and the developmental age of the CAM may not be the only factors which influenced the success rate in the present study. We speculate that the higher humidity levels of 71 to 74% during the *in ovo* culture period could have contributed to graft survival, since preliminary trials showed that using the standard 60% humidity level reported in all previous studies, that involved the *in ovo* culturing of ovarian tissue, resulted in partial keratinization of the CAM by 15 d of embryonic development (*unpublished data*). Keratinization has been linked to poor humidity conditions when the CAM is cultured *ex ovo* (Nowak-Sliwinska et al., 2014). It is unknown if CAM keratinization might reduce the number of blood vessels within it, and therefore reduce the potential revascularization of the graft. If it does, then keratinization might affect the integrity of the CAM culture system, this although has not been reported in previous *in ovo* culture studies. Keratinization was not observed at the higher humidity level used here, which to the best of our knowledge is the first time 71 to 74% humidity has been used during *in ovo* culturing of ovarian tissue. Meaning that it was removed as a possible environmental effect, although as previously mentioned, the high humidity might have been a factor in reducing the chicken embryo survival rate.

Tissue Morphology and Cellular Maturation

The gross morphology of grafts was similar for all ages, with prefollicular germ cells and follicles populating the cortical region (Figures 3E–H). In the 1 dph + *in ovo* grafts, however, there was a loss of definition between the cortex and medulla that was based on the lack of germ cells (Figure 3E). Although, lacunar channels were still visible within the medulla region of the ovarian tissue and large blood vessels were visible within the CAM surrounding the graft. One notable difference between grafts, irrespective of age group, was that some appeared to have CAM surrounding them (Figure 3E), whereas others appeared to only have CAM around their lower half (Figures 3F–H). This could be due to the level of CAM traumatization or the thickness of the CAM at the time of grafting, with thinner or more traumatized CAM leading to grafts sinking lower and allowing the membrane to heal over top of the transplanted segments.

The size of prefollicular germ cells and follicles within preculture, cultured, and *in vivo* ovaries were compared to determine if these cells grew during *in ovo* culture. In other words, growth would indicate that the tissue was developing on a cellular level and viable. There was no difference ($P > 0.05$) in prefollicular germ cell diameters between 1, 3, 11, and 15 dph + *in ovo* grafts, compared with their preculture counterparts (Figure 4A). By contrast, 5 and 7 dph + *in ovo* grafts had larger ($P \leq 0.002$) prefollicular germ cells (16.09 ± 0.48 μm; 17.89 ± 0.38 μm, respectively) compared with their preculture counterparts (13.30 ± 0.24 μm; 14.05 ± 0.16 μm, respectively). However, the size of the germ cells in the grafts was smaller ($P \leq 0.018$) than the germ cells within the comparable *in vivo* ovarian tissue (11 dph,

Table 2. Graft size and visual assessment of vascularization within turkey ovarian tissue after culturing *in ovo* for 6 d.

Ovarian age (dph)	Grafts cultured	Embryos survived	Graft size (mm^2)	Vascularization		
				Well/partly	Poor	Necrotic
1 + *in ovo*	12	11	3.3 ± 0.4[a]	11	0	0
3 + *in ovo*	15	10	4.5 ± 0.3[a]	10	0	0
5 + *in ovo*	12	12	4.0 ± 0.3[a]	12	0	0
7 + *in ovo*	15	13	4.2 ± 0.3[a]	13	0	0
11 + *in ovo*	15	14	4.3 ± 0.3[a]	14	0	0
15 + *in ovo*	15	14	4.7 ± 0.4[a]	14	0	0
Total	84	74	-	74	0	0

Also included are the number of ovarian grafts per age, and surviving chicken embryos used for *in ovo* culturing. Before culturing, the ovaries were collected from poults 1 to 15 dph. Graft size data are means ± SEM.
[a]Means with a common superscript do not differ significantly ($P > 0.05$).
Abbreviation: dph, days posthatch.

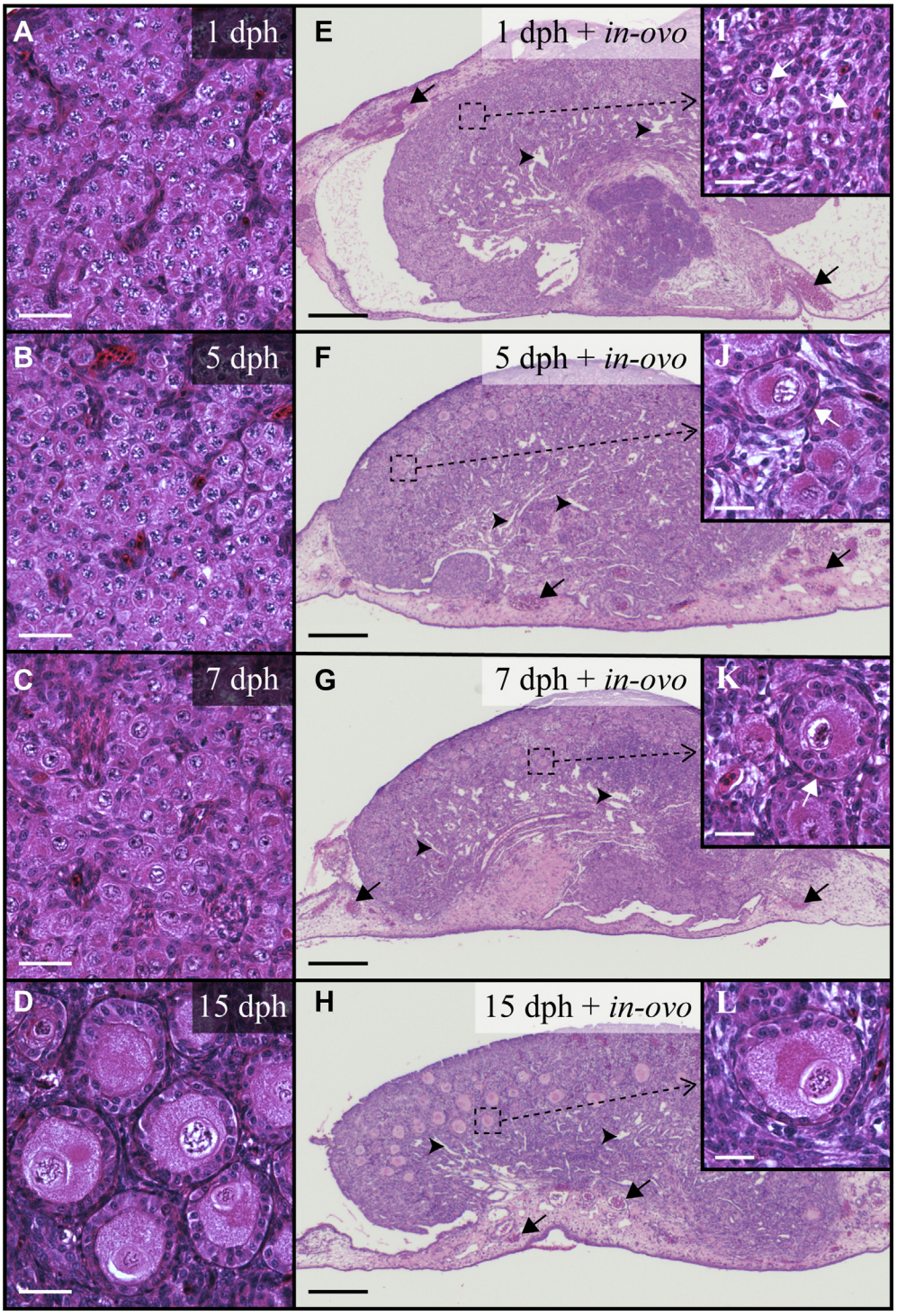

Figure 3. Histological appearance of turkey ovarian tissue preculture, and after culturing *in ovo* for 6 d. Images of the cortical area from preculture ovarian tissue at 1, 5, 7, and 15 dph are shown in A–D. In 1, 5, and 7 dph preculture tissue, the cortex is predominately made up of prefollicular germ cells, whereas at 15 dph, the cortex is populated with follicles. Images through the center of the grafts from 1, 5, 7, and 15 dph + *in ovo* are shown in E–H. Arrows indicate large blood vessels within the CAM close to the grafts, whereas the arrowheads show lacunar channels within the medulla portion of the grafts. In addition, I–L correspond to higher magnifications of the cortex of the grafts (dotted square). In 1 dph + *in ovo* (I) the prefollicular germ cells (arrows) are still present within the cortex although at a lower number. In 5 dph + *in ovo* (J) novel growing primordial follicles are now visible within the cortex (arrow), whereas in 7 dph + *in ovo* grafts (K), primary follicles can be seen (arrow). Finally, in 15 dph + *in ovo* grafts (L) follicles are present, although in lower numbers. Scale bars (A–D) 30 μm, (E–H) 200 μm, (I–L) 20 μm. Abbreviations: CAM, chorioallantoic membrane; dph, days posthatch.

18.21 ± 1.03 μm; 13 dph, 20.74 ± 0.54 μm). No differences ($P > 0.05$) in follicle diameters were observed between 7, 11, and 15 dph + *in ovo* grafts (41.23 ± 1.19 μm; 49.43 ± 1.59 μm; 56.18 ± 1.00 μm, respectively) and their preculture counterparts (37.48 ± 1.45 μm; 48.95 ± 1.18 μm; 53.95 ± 1.15 μm,

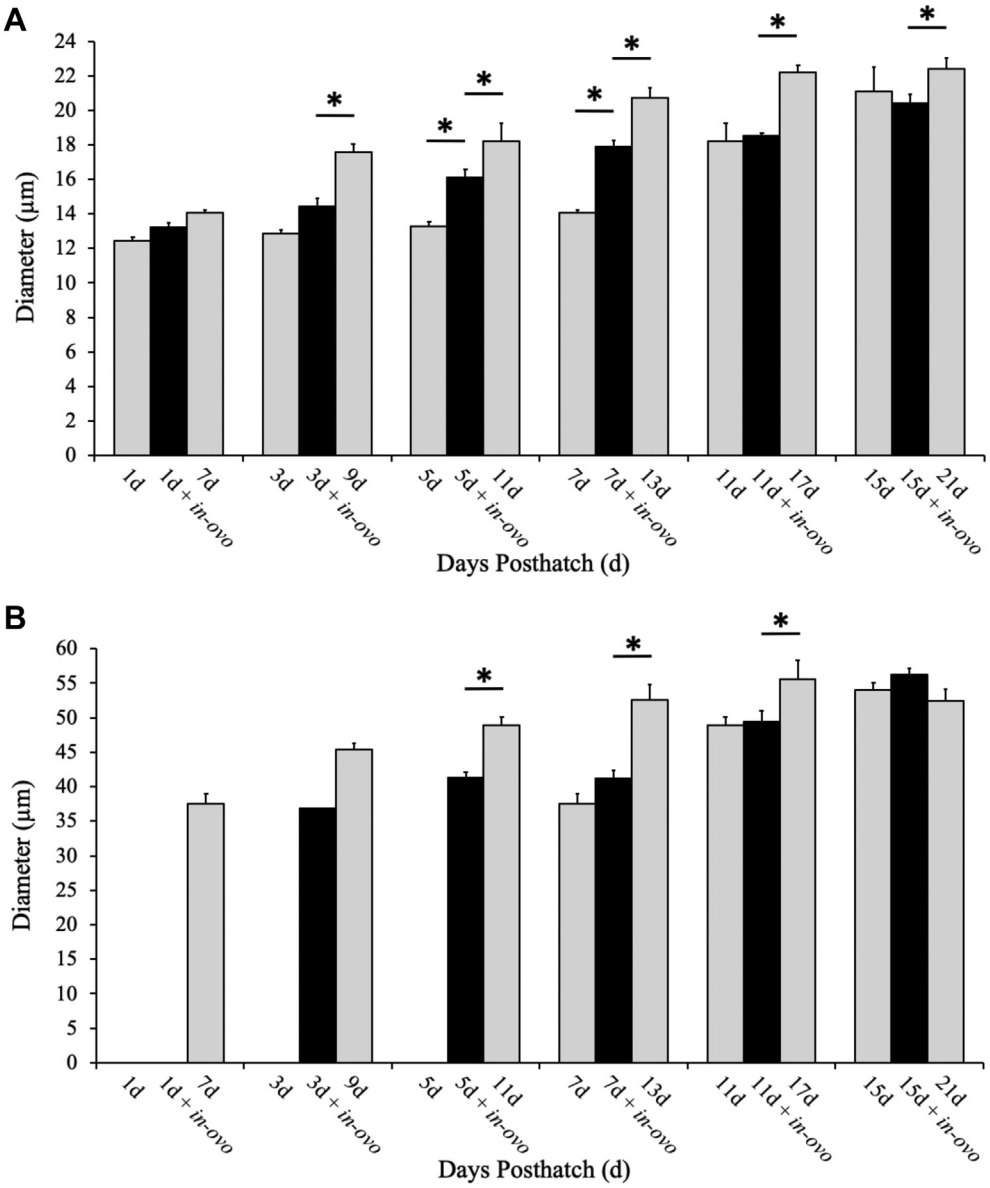

Figure 4. Prefollicular germ cells in turkey ovarian tissue can grow while cultured *in ovo* for 6 d; however, follicles do not. Prefollicular germ cell (A), and follicle (B) diameters within cultured ovaries (black), were compared with fresh ovaries (gray), with fresh ovaries being defined as preculture (gray bar to the left) or *in vivo* (gray bar to the right), based on their relative age. For 5 and 7 dph + *in ovo* grafts prefollicular germ cells grew based on the significant increase in size compared with their preculture counterparts (5 and 7 dph). There was no difference in follicle diameter within grafted ovaries compared with their preculture counterparts. Therefore, 5, 7, and 11 dph + *in ovo* grafts had smaller follicles compared with their *in vivo* counterparts (11, 13, and 17 dph). Data are means ± SEM of n = 4 (*$P \leq 0.05$). Abbreviation: dph, days posthatch.

respectively), as shown in Figure 4B. A comparison of 5 dph + *in ovo* grafts with their preculture counterpart (5 dph) was not possible as the preculture ovaries lacked follicles. However, follicles (41.32 ± 0.73 µm) within 5 dph + *in ovo* grafts were smaller ($P = 0.001$) than the ones in 11 dph ovarian tissue (48.95 ± 1.18 µm). Statistical analyses of follicle diameters in 3 dph + *in ovo* grafts were not possible as only one of the 4 ovaries contained follicles. It was clear that turkey prefollicular germ cells can grow in ovarian tissue cultured *in ovo*; however, there was no evidence that follicles can increase in size under the same conditions. By contrast, when cow ovarian tissue was cultured *in ovo* for 8 d, a small number of follicles were able to increase in size (Gigli et al., 2005). We are not aware of other studies investigating the growth of prefollicular germ cells in an *in ovo* culture system.

The density of prefollicular germ cells and follicles within preculture, cultured, and *in vivo* ovaries were analyzed to evaluate the changes in cell populations. Evaluating density to predict changes in prefollicular germ cell and follicle populations within turkey ovarian tissues has already been validated by our group (Hall et al., 2020). Based on this approach, a dramatic change was observed in prefollicular germ cell and follicle populations, along with the appearance of new follicles, between 5 and 7 dph + *in ovo* grafts and their preculture counterparts (Figure 3). Moreover, the prefollicular

germ cell density (116,025 ± 23,988/mm^3) within 1 dph + *in ovo* grafts was lower ($P \leq 0.001$) than *in vivo* (279,478 ± 52,239/mm^3) 7 dph counterparts (Figure 5A), indicating that at this age, prefollicular germ cell numbers decreased because of culturing *in ovo*. This could be due to an accelerated natural decline, or these particular germ cells being more susceptible to cell death at this age. Based on the size of prefollicular germ cells within 1 dph + *in ovo* grafts, the natural development of prefollicular germ cells does not appear accelerated. Therefore, this decline in prefollicular germ cell density is more than likely due to an increase in cell death.

No other differences ($P > 0.05$) in prefollicular germ cell densities were detected between the *in ovo* culture groups and the *in vivo* counterparts. However, a 4-fold numerical difference ($P = 0.141$) was observed between 7 dph + *in ovo* grafts (81,030 ± 17,611/mm^3) and their *in vivo* counterparts (13 dph, 16,463 ± 6,805/mm^3). Although not statistically significant, it could be biologically relevant as prefollicular germ cell development may have been disrupted as previously mentioned for 1 dph + *in ovo* grafts. In this case, the process might have been slowed down with fewer germ cells dying than normal through this transitional period. Once again, based on the size of prefollicular germ cells within 7 dph + *in ovo* grafts, it does appear as though the normal process of germ cell development was delayed, which resulted in more prefollicular germ cells being present at this age. This is the first evidence suggesting prefollicular germ cell development is affected by *in ovo* culturing, and this effect is age-dependent.

In terms of follicle densities, 1 dph + *in ovo* grafts did not contain any follicles, although they were present in 7 dph *in vivo* ovarian tissue (Figure 5B). Conversely, follicles were present in 3 dph + *in ovo* grafts, whereas none

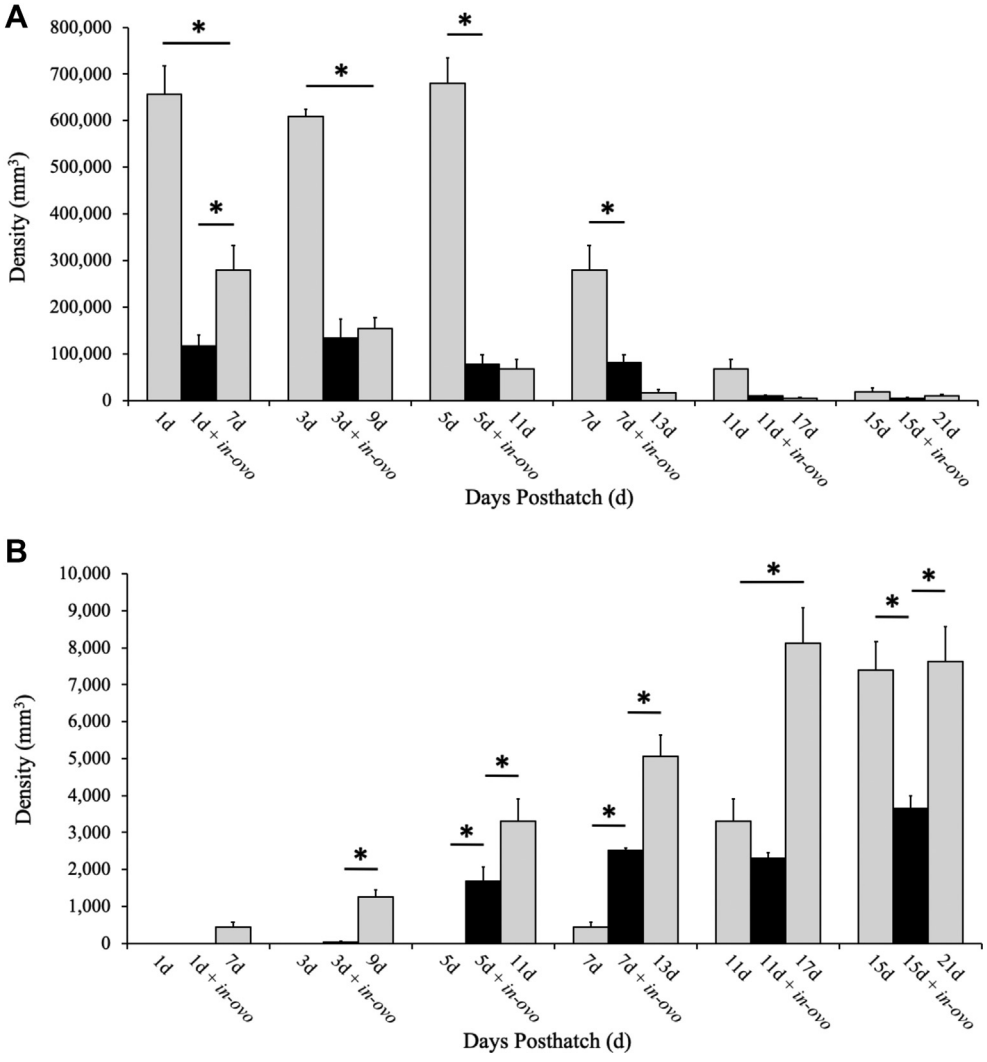

Figure 5. The donor age affects the outcome of prefollicular germ cell and follicle densities after culturing turkey ovarian tissue *in ovo*. Prefollicular germ cell (A), and follicle (B) densities within cultured ovaries (black) were compared with fresh ovaries (gray). Fresh ovaries are defined as preculture (gray bar to the left) or *in vivo* (gray bar to the right), based on their relative age. The density of prefollicular germ cells within 1 dph + *in ovo* grafts was lower than its *in vivo* counterpart (7 dph). Whereas, 7 dph + *in ovo* grafts had a 4-fold difference in its numerical value compared with its *in vivo* counterpart. For follicles within 5 and 7 dph + *in ovo* grafts, there was an increase in their densities compared with their preculture counterparts. However, 11 and 15 dph + *in ovo* grafts saw no change or a decrease compared with their *in vivo* counterparts. Data are means ± SEM of n = 4 (*$P \leq 0.05$). Abbreviation: dph, days posthatch.

were present in their preculture counterparts. In 5 and 7 dph + *in ovo* grafts, there was an increase ($P \leq 0.05$) in follicle densities (1,678 ± 384/mm^3; 2,511 ± 63/mm^3, respectively) compared with their preculture counterparts (0 ± 0/mm^3; 437 ± 125/mm^3, respectively). However, levels did not reach that of their *in vivo* (11 and 13 dph) counterparts. No change ($P = 0.118$) in follicle density was observed between 11 dph + *in ovo* grafts (2,301 ± 156/mm^3) and their preculture counterpart (3,315 ± 602/mm^3), whereas the density was considerably lower ($P < 0.001$) than in the 17 dph *in vivo* (7,398 ± 776/mm^3) counterpart. When 15 dph ovarian tissue was cultured, a large decrease ($P < 0.001$) in follicle density (3,655 ± 336/mm^3) was observed compared with preculture (7,398 ± 776/mm^3) and *in vivo* (7,633 ± 932/mm^3) counterparts. It appears that follicle formation or assembly did occur for the 3, 5, and 7 dph + *in ovo* grafts; however, the development was delayed compared with *in vivo* ovaries.

A delay in growth was also apparent from the size of the follicles. The follicle density in the 11 dph + *in ovo* grafts was not different from its preculture counterpart, suggesting that either additional follicles are not forming, or the rate of formation is counterbalanced by the rate of loss, resulting in no net change. As a decrease in follicle density was seen in 15 dph + *in ovo* grafts, it would appear that follicular death was accelerating in older grafts while follicular formation ceased. This finding suggests that follicles within turkey ovaries are susceptible to atresia at the time of grafting. It is likely then, that for 11 dph + *in ovo* grafts, the lack of change was a result of the formation of follicles being equal to the rate of loss. In mammalian studies, it was also demonstrated that follicles are lost during *in ovo* culturing (Vilela et al., 2016; Beck et al., 2018), as well as during allotransplantation under the kidney capsule (Candy et al., 1997). Therefore, the loss of follicles here is not confined to turkeys or the *in ovo* culture system, but rather transplantation of ovarian tissue in general. These losses potentially result from the ischemia and reperfusion injuries experienced by the ovarian tissue.

This is the first study reporting that follicles can develop from prefollicular germ cells and immature granulosa cells within an *in ovo* CAM culture system. While this transition has been relatively well documented *in vitro* for mammalian ovaries (Klinger and De Felici, 2002; Obata et al., 2002; Spears et al., 2003), the same cannot be said for birds. Previous reports on *in ovo* culturing of cow and mouse ovarian tissue provided little evidence that primordial follicles can transition into primary follicles (Cushman et al., 2002; Gigli et al., 2005). Here we saw primary follicles within 7 dph + *in ovo* grafts (Figure 3K), showing that this transition is also possible within avian ovaries cultured *in ovo*.

To determine the level of cellular death within cultured tissue and *in vivo* ovaries, a TUNEL assay was utilized to detect late-stage apoptosis. It is assumed that cellular damage from ischemia and reperfusion would have occurred by day 3 or 4 of culturing *in ovo* (Beck et al., 2018). After 6 d of culturing, any cells dying in response to the ischemic or reperfusion damage would be at the end of the apoptotic pathway. Overall, the percentage of late-stage apoptosis ranged from 0.3 to 0.8% (0.5 ± 0.1%) for *in vivo* ovaries (7–21 dph) and from 0.4 to 1.3% (0.8 ± 0.1%) for their *in ovo* cultured counterparts ($P = 0.051$). If cultured ovaries did have a slightly higher level of late-stage apoptosis, this small increase would more then likely not be biologically relevant. Nonetheless, the age of the tissue did not affect ($P = 0.091$) the level of late-stage apoptosis, neither did the interaction between age and type of ovary ($P = 0.610$). All values were substantially lower than the bursa of Fabricius (6.5 ± 0.3%), as shown in Figure 2C. The percentage of late-stage apoptosis within fresh ovarian tissue in our study was lower than what has been previously reported for fresh turkey ovarian tissue, collected from poults 1 to 7 dph (Liu et al., 2017). The reason for this difference is unclear; however, we can conclude that the TUNEL results here for the ovarian tissue were accurate. Based on the level of late-stage apoptosis detected within the turkey bursa of Fabricius being comparable with broiler studies, which used birds at similar ages (Peng et al., 2015; Yuan et al., 2016; Killian et al., 2017), where there is a larger body of research.

CONCLUSION

Significance and Impact for Avian Ovarian Transplantation

The overall goal for this project was to determine if donor age impacts turkey ovarian tissues transplantability, with the aim of recommending a suitable donor age for future studies. This could have been a possible factor that influenced the success rates in previous chicken, quail, and duck studies (Song and Silversides, 2008a, b; Song et al., 2011). Based on graft viability and percentage of late-stage apoptosis, all ages initially appeared suitable for transplantation; however, density results suggested otherwise. Ovarian tissue at 1 dph is not recommended based on the decrease in prefollicular germ cells during *in ovo* culturing, and ovarian tissue at 11 and 15 dph would also not be recommended based on the smaller pool of follicles and the depleted prefollicular germ cell reservoirs after culturing. Of the remaining ages (3, 5, and 7 dph), ovarian tissue from 7 dph poults potentially showed the highest prefollicular germ cell density compared with its *in vivo* counterpart, and at this age, prefollicular germ cells were able to grow significantly, demonstrating their viability. Therefore, we would recommend using 7 dph turkey ovarian for future transplantation studies.

If in poultry overall ovarian graft viability is not affected by the donor age, then it appears that the difference in success rates observed between the chicken, quail, and duck studies is a result of other factors (Song and Silversides, 2008a; b; Song et al., 2011). However, the 7 dph turkey ovarian tissue had a higher density of prefollicular germ cells after culturing, which

could indicate that on maturation these grafts would possess more viable germ cells. This could potentially lead to more donor-derived progeny or a higher ratio of donor- to host-derived. In fact, the quail study did see a higher ratio (173:57) of donor- to host-derived progeny within the immunosuppressed group; compared with a similar group in the chicken (351:611) study. Therefore, it appears that the increased number of surviving germ cells within the older tissue could potentially lead to a higher donor- to host-derived progeny ratio, meaning that donor age may still be an important factor to consider when transplanting poultry ovarian tissue.

In conclusion, the overall viability of turkey ovarian tissue cultured *in ovo* is not affected by age (1–15 dph) of the transplants; however, the survival of prefollicular germ cells and follicles within the transplanted ovarian tissue is. Of the ages tested, 7 dph tissue was recommended for future transplantation studies. Beyond *in ovo* culturing, the next step should focus on determining the factors that influence the ovarian tissue's ability to attach and revascularize in the abdominal cavity of a recipient poults. This stepwise approach to determining the best conditions for ovarian transplantation in the domestic turkey is important if the protocol is going to be consistent, and reproducible.

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