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Increasing Age Influences Uterine Integrity, but Not Ovarian Function or Oocyte Quality in the Cheetah (*Acinonyx jubatus*)*

Short title: Age influence on female cheetah reproduction
Summary sentence: Older female cheetahs undergo normal folliculogenesis, ovarian steroid production, ovulation, fertilization and early embryo development, with lower reproductive competence in these individuals due to compromised uterine integrity.
Key words: felid, aging, ovary, uterus, gamete
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Abstract

Although the cheetah routinely lives for >12 yr in ex situ collections, females >8 yr of age reproduce infrequently. We tested the hypothesis that reproduction is compromised in older cheetah females due to a combination of a disrupted gonadal, oocyte and uterine function/integrity. We assessed (1) ovarian response to gonadotropins, (2) oocyte meiotic, fertilization and developmental competence and (3) uterine morphology in three age classes of cheetahs (young, 2 – 5 yr, n = 17; prime, 6 – 8 yr, n = 8; older, 9 – 15 yr, n = 9). Ovarian activity was stimulated with a combination of eCG and hCG, and fecal samples were collected for 45 days before gonadotropin treatment and 30 days after oocyte recovery by laparoscopy. Twenty-six to 30 h post-hCG, uterine morphology was examined by ultrasound, ovarian follicular size determined by laparoscopy and aspirated oocytes assessed for nuclear status or inseminated in vitro. Although there was no influence of age on fecal hormone concentrations or gross uterine morphology ($P > 0.05$), older females produced fewer ($P < 0.05$) total antral follicles and oocytes compared to younger counterparts. Regardless of donor age, oocytes had equivalent ($P > 0.05$) nuclear status and ability to reach metaphase II and fertilize in vitro. A histological assessment of voucher specimens revealed an age-related influence on uterine tissue integrity with >87% and >56% of older females experiencing endometrial hyperplasia and severe pathologies, respectively. Our collective findings reveal that lower reproductive success in older cheetahs appears minimally influenced by ovarian and gamete aging and subsequent dysfunction. Rather, ovaries from older females are responsive to gonadotropins, produce normative estradiol/progestagen concentrations and follicles containing oocytes with the capacity to mature and fertilize. A more likely cause of reduced fertility may be the high prevalence of uterine endometrial hyperplasia and related pathologies. The discovery that a significant proportion of oocytes from older females have developmental capacity in vitro suggests that IVF and embryo transfer may be useful for 'rescuing' the genome of older, non-reproductive cheetahs.

Introduction

Numbers of cheetahs living in nature have decreased ~85% since 1900 [1-3], with the remaining ~10,000 individuals threatened by continuing loss of habitat and prey base [2, 3]. Thus, having a viable ex situ (captive) population is important (1) as a hedge against extinction, (2) for promoting public awareness and, most importantly, (3) for studying biological phenomenon virtually impossible to investigate in rare and elusive free-ranging individuals [4]. However, the cheetah always has been challenging to reproduce consistently in captivity. Since 1970, ~78% of females and ~82% of males in North American institutions have died without reproducing [5, 6]. To facilitate genetic management of this species in North American institutions, there is a group of 56 cooperating institutions that pool animals in a Species Survival Plan (SSP). This population, currently at ~210 adult cheetahs, has never been self-sustaining, and death rate has exceeded birth rate in 13 of the last 16 years [5, 6]. Approximately 85% of adult females in the current North American population has never reproduced [5]. Of females producing young for the first time, ~65% have been 2 to 5 y of age [5, 6]. Thirty-seven percent of females in this contemporary population already are ≥8 y old, with an average lifespan in captivity of ~12 y [6]. This longevity is at least 6 y longer than what has been measured in two wild populations [7, 8]. Thus, not only is overall reproduction poor, but the advanced age skew is ominous for this ex situ population's future. Consequently, a priority is understanding the impact of age on ovarian, oocyte and uterine function. Such information can hold clues to addressing suboptimal fecundity in cheetahs in general and specifically for older females that are genetically valuable but still under-represented in the population.

The female cheetah is known to be reproductively active throughout the year (cycles average 13.6 days in length [9]), although with unpredictable and variable intervals of ovarian quiescence [10]. This phenomenon has been observed widely on the basis of direct ovarian observations [11] and endocrine patterns and is unrelated to animal age because periods of ovarian shut-down occur even during prime reproductive years [9]. The species is an induced ovulator [9], and females ranging from 3 to 12 y of age have been shown to be highly responsive to a simple exogenous gonadotropin treatment to provoke follicular development and ovulation [12, 13]. Aspirated follicular oocytes have the capacity to achieve nuclear maturation and fertilize in vitro [14]. Artificial insemination also is more successful in younger (2 – 5 y old) than older sperm recipients [13].

What is known about age effects on mammalian ovarian and oocyte physiology comes from species distantly related to felids (for reviews see [15-18]). Specifically, for primate and murine models, there is an accelerated loss of oocytes near the onset of reproductive senescence occurring coincidentally with irregular and protracted cycles [17, 19], decreases in oocyte number [17, 19] and quality [20] and sharp declines in fecundity [21, 22]. Circulating FSH and LH concentrations also rise as the transition to senescence nears (human [23]; nonhuman primates [24, 25]) while estrogen [24, 26] and progesterone concentrations [26] decrease markedly. More specific studies have indicated that advancing age influences both nuclear and cytoplasmic factors critical for oocyte maturation, fertilization and embryo development. For example, oocytes from older murine and human females experience more oocyte DNA fragmentation, meiotic incompetence and compromised fertilization compared to younger counterparts [20, 27, 28].

There is minimal information on the influence of age on reproductive function in any carnivore species. For the domestic dog, a greater number of high quality oocytes are recoverable from the ovaries of adult bitches (2 – 6.5 y of age) compared to gametes recouped from very young (45 – 60 d) or older (>9.5 y) donors [29]. In colony-reared domestic cats, aging appears to markedly influence uterine integrity. In one study, ~88% of females >5 y of age were experiencing cystic endometrial hyperplasia compared to an incidence of only 30% in 2 to 4 y old individuals [30]. Similarly, a survey

of 212 wild felids representing 23 species managed in zoos has revealed more endometrial hyperplasia in females that are older, have a history of nullipary and/or exposure to exogenous progestins (for contraceptive purposes) [31].

Our general aim was to begin understanding the influence of age on reproductive function in the cheetah, specifically exploring if there was an effect on ovarian function/activity, oocyte quality/developmental competence and uterine morphology/integrity. We hypothesized that all three of these reproductive components were being influenced by the aging process, especially in females that were 9 y or older. Our specific objectives were to measure: 1) ovarian responsiveness over time, non-invasively via fecal steroid metabolite patterns and then laparoscopically post-gonadotropin treatment; 2) oocyte quality, including ability to achieve maturation, fertilize and develop to early embryo stages in vitro; and 3) uterine status by evaluating living females via ultrasonography as well as histological analysis of previously collected specimens. Thus, our comparative approach targeted three components of the reproductive system (gonad, gamete, uterus) and was bolstered by our unique, special access to significant number of individuals of this endangered species across three distinctive age classes. This included availability of some cheetahs of wild-caught origin living in Namibia, Africa.

Materials and Methods

Animals

All animal-related procedures were approved by the National Zoological Park's Institutional Animal Care and Use Committee (IACUC) and similar committees of the Birmingham Zoo, White Oak Conservation Center, Denver Zoological Gardens, Living Desert, Wildlife Safari, Fossil Rim Wildlife Center and Cheetah Conservation Fund (CCF), Namibia. Adult females (2 – 15 y of age, n = 29 total) were managed either singly or in groups at these North American institutions (all participants within the SSP, n = 12 cheetahs) or at CCF (n = 17). The latter is a nongovernmental organization near Otjiwarongo, Namibia, dedicated to studying and conserving this species in that range country. Five females at the CCF facility were included in the study protocol twice with a 1 y interval between procedures. Cheetah age was estimated by a thorough examination of tooth wear [32].

Animals were assigned to one of three age category groups: 'young' (2 – 5 y, n = 17), 'prime' (6 – 8 y, n = 8) or 'older' (9 – 15 y, n = 9). Justification for this classification scheme was based on information gleaned from ex situ breeding and studbook records for the North American cheetah population [6]. Specifically, the majority (~65%) of first time cheetah pregnancies were established in females from 2 to 5 y of age with ~25% of initial conceptions taking place from years 6 through 8. There was a steep decline in reproductive competence in females that were 9 y or older with <5% of first pregnancies occurring during this period [5, 6].

For females in the current study, 100% (17 of 17) of the young, 75% (6 of 8) of the prime and 44% (4 of 9) of the older females were nulliparous. For each age group, we evaluated (1) ovarian function and activity on the basis of endocrine patterns (assessed noninvasively by measuring estradiol and progestagen metabolites in feces) and direct ovarian observations of follicle number and morphology after gonadotropin treatment, (2) gamete quality as determined by subjective quality grade and objective capacity to achieve nuclear maturation, fertilization and early embryo development in vitro and (3) uterine status on the basis of gross appearance/morphometry as assessed by ultrasonography. Additionally, to generate a more detailed understanding of the role of age and uterine integrity in this species, we conducted a detailed histological evaluation of fixed tissue from known age, voucher specimens.

Ovarian function

Ovarian activity was monitored directly and indirectly. For the former, each ovary was examined thoroughly by laparoscopy [11, 13] after stimulation with an established exogenous gonadotropin regimen for this species [12, 13]. In brief, this treatment involved an i.m. injection of 300 IU of eCG followed 82 to 86 h later by 150 IU of hCG i.m. (Sigma Chemical Co., St. Louis, MO). Females were anesthetized using a combination of ketamine HCl (2.0 – 3.5 mg/kg body weight; Ketaset, Fort Dodge Laboratories, Fort Dodge, IA) and medetomidine hydrochloride (22 – 25 µg/kg; Domitor, Pfizer, Inc., La Jolla, CA) or a combination of the latter drug (40 µg/kg), butorphanol (0.3 mg/kg; Dolorex, Intervet, Inc., Millsboro, DE) and midazolam (0.2 mg/kg; Baxter Healthcare Corp., Deerfield, IL), all delivered i.m. 25 to 30 h post-hCG. After endotracheal intubation, anesthesia was maintained with isoflurane gas/oxygen inhalation as previously described [13]. After insufflation of the abdominal cavity with an ancillary Verres device, a 10 mm diameter laparoscope (Olympus Co., Melville, NY) was inserted along the abdominal midline to methodically examine both uterine cornua and all aspects of each ovary [11, 13]. A video camera (Olympus Co., Melville, NY) attached to the laparoscope was used to record ovarian and uterine horn external morphology to allow further assessments, as necessary. The known diameter of the Verres device allowed measuring the width and length of each ovary, with values then used to calculate volume for each gonad [(4 x π x 0.5 height x 0.5 length x 0.5 width)/3)] and then an overall combined ovarian volume per animal [33]. The Verres needle also was used to determine the length, width and height of each follicle to generate an estimated 'surface volume'. Because level of vascularization in the follicle wall is indicative of late preovulatory status and containment of a high quality oocyte in domestic cats [34], this metric was scored on a scale of 0 (no evidence of surface vessels; example, Fig. 1A) to 3 (expansive, highly evident vascularity; example, Fig. 1B), with scores then averaged for each female.

Ovarian function also was assessed in a subset of females (n = 16; 6 young, 6 prime, 4 older) by measuring fecal hormone metabolite concentrations before, during and after the eCG/hCG provocation. This was accomplished by collecting freshly-voided feces (within 24 h of excretion) once daily (4 to 5 days per week) for 45 days before gonadotropin stimulation and 30 days after oocyte recovery. Samples were stored frozen at –20°C and then shipped to the Smithsonian Conservation Biology Institute for analysis. Approximately 0.2 g of dried fecal powder were boiled in 90% ethanol:10% distilled water [9]. Each sample was centrifuged at 500 x *g* for 20 min, the supernatant recovered and the resulting pellet re-dissolved in 5 ml of 90% ethanol before re-centrifugation (500 x *g*, 15 min). This secondary supernatant was recovered, pooled with the first, dried under air and re-dissolved in 1 ml methanol (100%). Fecal extracts were vortexed, then sonicated for 15 min and stored at –20°C until hormonal analysis.

Enzyme immunoassays (EIAs) for measuring estradiol and progestagen metabolite concentrations were validated for cheetah feces by demonstrating: (1) parallelism between dilutions of pooled fecal extracts and the respective standard curve (1.95 – 500 pg/well; $y = 1.104x - 1.473$, $r = 0.99$ and 0.78 – 200 pg/well; $y = 1.173x - 3.153$, $r = 0.99$ for estradiol and progestagens, respectively); and 2) significant recovery of exogenous estradiol (93%) or progestagens (85%) added to extracts before analysis [35]. Fecal estradiol concentrations were quantified using a polyclonal antibody assay (No. R4972, C. Munro, University of California, Davis, CA) and associated horseradish-peroxidase ligand. The inter-assay coefficients of variation (CV) for two internal controls (n = 46 assays) were 11.2% and 10.0%, and the intra-assay CV was <10%. Progestagen metabolite concentrations were quantified using a monoclonal antibody assay (No. CL42, Quidel Co., San Diego, CA) and associated horseradish-peroxidase ligand [36]. The inter-assay CVs for two internal controls (n = 64 assays) were 12.9% and 17.2%, and the intra-assay CV was <10%. The EIAs were performed in 96-well microtiter plates (Nunc-Immuno, Maxisorp Surface; Fisher Scientific, Pittsburgh, PA) with assay-specific

standards (Steroloids, Inc., Newport, RI) and diluted fecal extracts assayed in duplicate. Means for steroid hormones were calculated for individual animals for: Days -45 through 0 (pre-eCG/hCG); the 5 day peri-eCG/hCG interval (Day +1 – +5 with Day +1 = day of eCG injection); and for periods Days +6 – +16, Days +17 – +26 and Days +27 – +36. Mean hormone values were pooled for each of these five intervals within the three age groups.

Oocyte assessments, fertilization and embryo development

At the time of laparoscopy and after quantifying ovarian metrics, oocytes were aspirated from antral follicles (≥2 mm diameter [11]) with a 22-gauge needle inserted through the abdominal wall and attached to an aspiration device [37]. Recovery efficiency (number of oocytes collected/number of follicles aspirated) for all females combined was 91.7 ± 8.2% (no difference, $P > 0.05$, among age groups). Oocytes were pooled and graded according to standard criteria for felids [38]. Briefly, Grade 1 ('excellent') oocytes had a uniformly dark cytoplasm and multiple layers of expanded cumulus cells (Fig. 1C). Grade 2 ('good') oocytes had a similar cytoplasmic appearance, but with fewer layers of expanded cumulus cells. Grade 3 ('average') oocytes had a slightly vacuolated and non-uniform cytoplasm as well as few (1 – 3) layers of cumulus cells. Grade 4 ('poor') quality oocytes were characterized by vacuolated and inconsistent cytoplasm as well as a lack of cumulus cell investment.

A subset of oocytes (n = 1 – 4) from each female was fixed in 2.5% paraformaldehyde and stored at 4ºC before microtubule immunostaining [39]. In brief, non-specific sites within the oocyte were blocked by incubation in 0.5% solution of Triton X100 in PBS with 20% FCS. Oocytes were incubated overnight at 4ºC with anti-α-tubulin monoclonal antibody (Sigma Chemical Co.) diluted 1:2,000 in blocking solution and then incubated with FITC-labeled anti-mouse IgG diluted 1:150 for 1 h at 38ºC. Chromatin was counter-stained with 5 µg/ml Hoechst 33342 for 5 min at 38ºC [39]. After mounting on a slide with Vectashield medium (Vector Laboratories, Burlingame, CA), oocytes were evaluated using epifluorescence [39]. Figure 1 depicts cheetah oocytes at anaphase I (Fig. 1D), telophase I (Fig. 1E) and metaphase II (Fig. 1F) of maturation.

To assess fertilization and subsequent development, 282 oocytes (152 from young; 80 from prime; 50 from older females) were inseminated in vitro with conspecific spermatozoa that previously had been collected by electroejaculation and cryopreserved [11, 40-42]. Five ejaculates from four anesthetized [41-43] males were evaluated immediately for seminal volume and sperm concentration (using a hemacytometer), sperm motility and forward progressive status [40, 41]. Samples were diluted, washed and cryopreserved in plastic straw containers over liquid nitrogen vapor [42, 43]. Straws were individually thawed for 10 sec in air followed by 30 sec in a 37˚C water bath, evaluated to ensure adequate sperm motility metrics and concentration (see below), diluted in HF10 (500 µl per straw) and centrifuged (100 *g*, 8 min) [42, 43]. After aspirating the supernatant and swim-up processing (60 µl of fresh HF10 for 30 min at ambient temperature [43]), ~50 µl of sperm suspension was recovered and concentration and motility assessed.

Oocytes were inseminated in 50 µl microdrops of HF10 without Hepes supplemented with pyruvate (1 mM), L-glutamine (2 mM), 10,000 IU/ml penicillin and 10 mg/ml streptomycin (Sigma Chemical Co.) under mineral oil (38.5°C; 5% CO_2 in air) with ~4.0×10^6 motile sperm/ml (from one aliquot of thawed ejaculate as processed above). Additionally, from one to four oocytes per female were incubated in medium without sperm (as parthenote controls). The average (± SEM) sperm motility immediately post-thaw for samples utilized for in vitro insemination of cheetah oocytes was 46.3 ± 2.5%. After swim-up separation, the proportion of motile cells increased ($P < 0.01$) to 68.8 ± 1.4%, and the average total motile sperm used for each insemination was $1.9 \pm 0.5 \times 10^5$. The proportions of

sperm with normal morphology and intact acrosomes were 36.5 ± 3.3% and 30.5 ± 5%, respectively, after swim-up processing. At ~18 h post-insemination (hpi), cumulus cells and bound spermatozoa were removed by mechanical stripping. Presumptive zygotes then were transferred to a fresh medium droplet and cultured in HF10 in the same in vitro environment for an additional 7 days. The culture medium was not changed during the 7 days. Uncleaved embryos were removed from culture and fixed at 32 hpi and nuclear status evaluated [44, 45]. Retrospective analysis of oocyte maturation or successful fertilization was determined through visualization of the nucleus or imaging of pronuclei post-staining. In brief, the latter involved evaluation of chromatin status with epifluorescence microscopy after fixation in ethanol and staining with Hoechst 33342 (Sigma Chemical Co.). At day 7 of culture (day 8 post-insemination), embryos earlier than the blastocyst stage of development were evaluated with epifluorescence microscopy after fixation in ethanol and staining with Hoechst 33342 to determine cell number [44, 45]. A morula was defined as an embryo containing 16 to 50 cells with no apparent blastocoele, and a blastocyst was an embryo with >50 cells and a discernible blastocoele by day 7 of culture

Uterine morphology and histology

Uterine status was evaluated by two means, one being by ultrasonography during the anesthesia interval for laparoscopic ovarian observation and oocyte aspiration. The uterine cornua also were observed directly during laparoscopy, but no morphological differences were apparent. Ultrasonography is well-established for quantifying ovarian morphology and ovulation timing, width and thickness of the uterine body/cornua and diagnosing uterine pathologies in felids [46]. For the present study, we used a Mindray 6600 digital diagnostic imaging system (Shenzhen Mindray Biomedical, Nanshan Shenzhen Cn, China) and a linear (5.0/7.5/10 mHz) scanhead to measure uterine body width, uterine body wall thickness, left and right uterine cornu total width (representative ultrasound image for assessment of cornu width; Fig. 2A) and left and right cornu wall thickness (representative image to assess wall thickness, Fig. 2B).

Our second approach for assessing relatedness of age to uterine status involved a histological assessment of biopsy specimens collected at necropsy from 1987 to 2008. There were 118 of these voucher specimens, which were distributed across our age categories as follows: young (2 – 5 y), n = 21; prime (6 – 8 y), n = 18; older (9 – 17 y), n = 79. Of the total, 105 samples (~89%) were derived from cheetahs that were captive-born and managed in the North American SSP population with the remaining 13 specimens (~11%) originating from individuals either free-ranging or captive-held (at CCF) in Namibia. Additionally, for these 118 voucher specimens, ~95% (20 of 21) of samples from young, ~72% (13 of 18) from prime and ~60% (47 of 79) of samples from the older age group were derived from nulliparous females. As described above, tooth wear [32] was used to age cheetahs in our Namibian study population. In all cases, each reproductive tract was evaluated for tissue structure, evidence of hyperplasia [31], and then two transverse sections of freshly-excised uterine endometrium (and any gross lesions) were fixed in 10% neutral-buffered formalin. Tissues were embedded in paraffin, sectioned at 7 μm, stained with hematoxylin and eosin and then examined histopathologically as previously described [31]. The severity of endometrial hyperplasia was categorized as: grade 0 = no hyperplastic changes (Fig. 3A); grade 1 = minimal-to-mild proliferative and/or cystic changes in glands or surface epithelium without an increase in overall endometrial height (Fig. 3B); grade 2 = moderate hyperplastic and/or cystic change with an increased endometrial thickness of ≤2 times normal (Fig. 3C); and grade 3 = severe hyperplastic and/or cystic changes with increased endometrial thickness of >2 times normal (Fig. 3D) [31]. All tissues were additionally categorized for the presence and type of severe pathology expressed, including cysts within the endometrium, endometrial fibrosis, hydrometra, adenomyosis, chronic lymphocytic endometritis, endometrial atrophy, hydrometra with

endometrial atrophy, pyometra and endometrial polyps [31]. The proportion of cheetahs experiencing hyperplasia and severe pathologies was plotted across the three age categories.

Statistical analysis

Data for ovarian morphology (assessed by laparoscopy), hormone metabolite concentrations, oocyte quality, embryo development and uterine morphology (evaluated by ultrasound) were analyzed by Pearson correlations and GLM procedures of SAS [47]. All percentage data were arcsine transformed before analysis. Means were separated using Duncan's multiple-range test. The final model for data on ovarian morphology, including total follicles and oocytes recovered as well as oocyte quality (maturation stage at aspiration) included the main effects of female age. The final model for analysis of hormone concentration data for each time interval (pre-eCG/hCG, peri-eCG/hCG, Days +6 – +16, +17 – +26 and +27 – +36) included the main effect of cheetah age group. For analysis of egg quality, the proportion of oocytes in metaphase II per female was determined based on total number of aspirated oocytes. The proportion of fertilized oocytes per female then was calculated based on total number of metaphase II stage oocytes for that individual. The proportion of cleaved embryos was determined based on the total number of fertilized oocytes per female. Finally, the proportion of embryos reaching 8 and 16 cells was determined on the total number of cleaved embryos for an individual female. The final model analyzing oocyte fertilization, cleavage and late embryo development included the main effects of female age and the covariate of ovarian volume. The final model for data on uterine morphology assessed via ultrasound, including the incidence of cysts and hydrometra, incorporated the main effects of age group and whether a female had previously given birth.

Proportional data for uterine histology metrics were pooled across age group and analyzed by Pearson correlations and non-parametric (NPAR1Way) procedures of SAS [47]. The class variables analyzed included age group, proven breeding status and the interval in years from last litter produced to animal death. Means were considered statistically different at $P < 0.05$, and results are reported as means ± SEM.

Results

Ovarian function

Age had no effect ($P > 0.05$) on average size of left or right ovary (data not shown) or total ovarian volume (Table 1). Follicle vascularization scores were similar ($P > 0.05$) across groups, but young cheetahs produced more ($P < 0.05$) total follicles (≥2 mm diameter) and a higher average number of recovered oocytes than older counterparts (Table 1). When females of all ages were combined, total ovarian volume for a given individual was correlated to total antral follicle number ($r = 0.47$, $P < 0.01$) and total oocytes recovered ($r = 0.50$, $P < 0.01$).

Interestingly, based on fecal estradiol concentrations, all females (for all age groups combined) had at least one estrous cycle during the six week sampling period prior to gonadotropin stimulation. Fecal estradiol and progestagen metabolite concentrations did not differ ($P > 0.05$) among age groups before, during the eCG/hCG peri-delivery interval or during the three post-ovulatory intervals examined (Fig. 4A-J). For all age groups combined (Fig. 4), estradiol values were highest ($P < 0.05$) during the peri-eCG/hCG interval compared to all other periods, with concentrations of this hormone already basal by the +6 to +16 day interval and continuing thereafter. Progestagen concentrations were basal and similar ($P > 0.05$) in all cheetah groups during the pre- and peri-eCG/hCG intervals, with the first detected increase occurring at +6 to +16 days with elevations again (and then stability) at +17 to + 26 days and thereafter (Fig. 4).

Oocyte assessments, fertilization and embryo development
When the total of 222, 98 and 77 oocytes recovered from young, prime and older cheetahs, respectively, were evaluated, the proportions meeting high quality grade criteria were similar ($P > 0.05$; Table 1). For all cheetahs combined, ~6% of oocytes (3 of 49) fixed at the time of aspiration demonstrated abnormal spindle formation, all recovered from two individuals in the young group.

The number of oocytes inseminated from young, prime and older females was 152, 80 and 50 oocytes, or 282 total of which 277 (98.2%) were quality Grade 1-2, and five (1.8%) were Grade 3. Because Grade 1 and 2 felid oocytes have comparable fertilization and developmental potential in vitro [37], gametes meeting these grades were pooled for insemination. The five Grade 3 oocytes were recovered from two prime age females.

The proportion of oocytes (those assessed at the time of aspiration combined with those evaluated at 32 hpi) that could be categorized as already at the metaphase II stage was comparable ($P > 0.05$) among age groups and ranged from 46 to 54% (Fig. 5). Likewise, the percentages of oocytes that were at the metaphase I, germinal vesicle or degenerate stage were similar ($P > 0.05$; Fig. 5). None of the parthenote controls were activated following culture in vitro. Overall, ~70% of all metaphase II oocytes cultured with sperm fertilized in vitro, an incidence of success that was similar ($P > 0.05$) among age groups (Table 1). Twelve of 282 total oocytes (4.3%) inseminated experienced polyspermy with no differences ($P > 0.05$) among groups (young, 3.9%; prime, 6.3%; older, 2.0%). More than half of all metaphase II oocytes from each age classification fertilized (mean range, 53.6 – 68.4%) with no differences ($P > 0.05$) among groups (Table 1). We observed a total of 27 morulae (Fig. 6A) and six blastocysts (Fig. 6B, C). Overall, ~34% of all fertilized and 41% of cleavage stage embryos progressed to at least 8 cells of development in vitro; ~24% of all cleaved embryos developed to the morula stage. The largest proportion of cleaved embryos developing to the morula stage in vitro occurred in the older compared to the prime and youngest groups (Table 1). All blastocysts were produced from oocytes aspirated from two young females.

Uterine morphology and histology
There was no influence ($P > 0.05$) of female age on any of the metrics measured via ultrasound (Table 2). Evidence of endometrial cysts was apparent in the uterine cornua or body of two cheetahs in the young (ages, 3 and 5 y), one in the prime (6 y old) and one in the older (15 y) group. Age had no influence ($P > 0.05$) on prevalence of endometrial cysts in adult cheetahs, with an overall prevalence of ~12% for the study cohort (Table 2). Three of the young females (3, 3 and 5 y old), two of the prime (6 and 7 y of age) and one of the older individuals (9 y old) had appreciable fluid (hydrometra) within the uterine cornua (Table 2), but this condition was unrelated ($P > 0.05$) to age. Additionally, none of the metrics displayed in Table 2 differed ($P > 0.05$) for females on the basis of pregnancy history; the frequency of occurrence was comparable between individuals that had given birth versus those that were nulliparous (data not shown).

Based on the histological assessments, the prevalence of uterine hyperplasia (all grades combined) increased ($P < 0.05$) with age, being 19% for young, 50% for prime and 87% for the older groups. Age also influenced ($P < 0.01$) the incidence of grade of hyperplasia; for example, Grade 3 hyperplasia (the most serious and advanced disease observed for this dataset) was measured in 19% of older cheetahs, but none of the young or prime females. Of the young cohort, only 19% expressed any form of hyperplasia, all being the least serious Grade 1. By contrast, Grade 1 and 2 hyperplasia was measured in 50% and 5.5%, respectively, of the prime and 44% and 28%, respectively, of the older age counterparts. The severe pathologies identified histologically included cysts within the endometrium,

endometrial fibrosis, hydrometra, adenomyosis, chronic lymphocytic endometritis, endometrial atrophy, hydrometra with endometrial atrophy, pyometra and endometrial polyps. The prevalence of severe uterine pathologies was highly age-dependent ($P < 0.0001$) with anomalies observed in only ~5% of young (n = 1), ~22% of prime (n = 4) and ~56% of tissues analyzed from the oldest cheetahs (n = 44) (Fig. 7). By far, the most common pathologies were adenomyosis (13% of all females), hydrometra (12%) and pyometra (7%), and often these anomalies occurred in combination. It was not unusual for individuals from the older group to experience multiple pathologies simultaneously at necropsy. For example, seven of the 79 older females [~9%] had a combination of hydrometra with accompanying adenomyosis or endometritis, adenomyosis with pyometra or fibrosis, and one female with a combination of polyps, cysts within the endometrium, hydrometra and adenomyosis. Again, reproductive history (parity versus nullipary) had no influence ($P > 0.05$) on uterine condition and integrity. However, the longer the time interval (in years) from the age a proven female last gave birth to her death, the more likely ($r = 0.68$, $P < 0.05$) that individual expressed a Grade 2 or 3 hyperplasia as well as severe uterine pathologies ($r = 0.57$, $P < 0.05$).

Discussion

Although the influence of aging has been only modestly studied in animal reproductive science, this topic is particularly relevant to ex situ collections of rare wildlife species. As significant advances have been made in husbandry and geriatric medicine, security populations of endangered species now live longer, and there is a need to know reproductive capacity in the context of lifespan [31, 48]. This is especially important for managed populations where the goal is to retain maximal genetic diversity through carefully planned breeding programs [4, 49]. In such instances, the most valuable individuals are matched for propagation, whereas senescent animals are retired from breeding to be used for public education in zoos. For species relying on such intensive management, there also is the need to understand which component(s) of the reproductive system fails and at what age, especially in instances where assisted breeding technologies could help ensure production of young from 'under-represented' individuals [4, 50]. In the case of older female cheetahs, we presumed that there would be a concurrent decline in ovarian function, oocyte quality and uterine competence over time, thereby explaining reduced fecundity in older individuals. Interestingly, although producing fewer ovarian follicles and recoverable oocytes, the older cohort was surprisingly normal in ovarian function and gamete quality based on our metrics. The influence of age within the time spans evaluated had minimal impact on steroidogenesis, grade of oocyte produced, maturational ability, fertilization or early embryogenesis in vitro. In contrast, age had a significant impact on uterine integrity that, although undetectable using ultrasonography, was evident from histological assessments of voucher specimens. Therefore, we concluded that reduced fecundity routinely observed in older cheetah females was caused largely by a high prevalence of uterine endometrial hyperplasia and related serious pathologies and not necessarily to ovarian or gamete dysfunction.

Our approach was thorough in that we compared functionality and integrity at three levels – gonad, oocyte and uterus – and, despite the species' rarity, across a significant number of individuals. Our strategy also was bolstered by having significant biological information on the cheetah, probably the most studied 'wild' animal in the reproductive sciences [4]. Besides extensive physiological data on sperm form and function [41, 42, 51, 52] and endocrine patterns (gonadal [9, 10]; adrenal [10, 53, 54]), there are substantial data on ovarian morphology, activity and sensitivity to exogenous gonadotropins [11-13]. Because the cheetah is an induced ovulator [9], and due to inexplicable and often protracted intervals of ovarian quiescence [9], the ovary of this species generally responds well and consistently to the eCG/hCG regimen used in the present study [12, 13]. Although producing only half the number of mature follicles measured in young or prime counterparts, follicles from older females were comparable in morphology (including vascularization) and contained equivalent quality oocytes and

fertilization capacity (see below). Likewise, there were no discernible differences in mean excreted estrogen during the peri-gonadotropin treatment interval (or at other periods), thereby suggesting normative folliculo-estrogenic activity across age groups. Similarly, luteal function as measured by mean progestagens was comparable, especially during the post-aspiration interval, indicating that steroid excretion capacity after a gonadotropin challenge was unaffected by age. Clearly, the ovaries of older female cheetahs had retained significant sensitivity to both an FSH and LH stimulus because follicles were provoked to develop, and oocytes were recoverable that later achieved nuclear maturation and fertilization in vitro. The reduction in follicle number (and naturally fewer recovered oocytes) in the older group logically may have been due to an age-related modest loss in gonadotropin receptivity in the ovary or, more likely, simply to fewer available, healthy follicles.

Our findings that the ovaries of older cheetahs are responsive to gonadotropins help explain the observations of behavioral estrus in aged animals, even individuals up to 12 y of age [10]. While a portion of the historically poor reproductive success of this species in captivity has been attributed to subpar management [10] and/or incompatibility within a breeding pair [11], clearly age also is a factor. Record assessments have determined that females 9 y of age or older have produced only 10% of the litters born since 1980 in accredited North American institutions attempting [6]. Additionally, less than 5% of females become pregnant for the first time after the age of 8 y [6]. Our findings of fewer ovarian follicles and oocytes recovered in older cheetahs also were compatible with earlier observations that animals in this age group consistently produce smaller-size litters (~2 cubs each) compared to younger counterparts (~4 cubs each [6]). Of course, this difference also could be related to the common discovery of a more compromised uterine environment in older individuals.

Indeed, there was strong evidence that, as the cheetah aged, the uterus became inhospitable, including by as early as 2 y (one young individual with hydrometra and endometrial atrophy). Interestingly, only a modest number of uterine abnormalities were observed via ultrasonography and even fewer by direct laparoscopic observations that, of course, only permitted surface evaluations of the cornua and uterine body. Retrospective histology of appropriately stored voucher specimens from necropsied cheetahs (generally housed originally at the same institutions as the living counterparts) was a more useful source of information. Clearly, older animals had significant more evidence of uterine anomalies than counterpart groups. Even so, half of prime age females were experiencing endometrial hyperplasia compared to more than 85% of the older age group. Histological assessments were even more enlightening in identifying a complexity of individual and combined serious pathologies, some of which may have eventually been diagnosed by ultrasonography in a well advanced stage [46]. The types and severity of these conditions likely prevent implantation and/or sustaining an early stage nidation [31].

Endometrial hyperplasia has been most thoroughly studied in humans [55] and, to a limited extent, in companion animals [56] and, in both cases, is known to lead to endometrial dysfunction and possibly sterility [31]. Endometrial hyperplasia in ex situ populations of wild felids has been recognized for years, but generally related to the protracted use of an exogenous progestin (melengestrol acetate, MGA) as a contraceptive to prevent reproduction in already well-represented individuals [31, 57, 58]. In one study of 212 zoo-managed specimens representing 23 felid species, endometrial hyperplasia was prevalent with more than 70% of all females demonstrating some degree of this pathology and almost 60% of those cases being considered severe [31]. In the latter study, cases of moderate-to-severe cystic endometrial hyperplasia were found in 85% of individuals implanted with MGA for a minimum of 6 mo [31]. The causative mechanism is believed to have been uninterrupted, high concentration progestin exposure that induces secretory differentiation of endometrial epithelial cells [31]. This hyperstimulated increase in cell number and differentiation causes cystic and adenomatous

changes characteristic of hyperplasia (hyperchromasia, pseudostratificaiton and a high nuclear/cytoplasmic ratio) [31]. However, our study population of cheetahs was never exposed to exogenous MGA or another progestin. Furthermore, as this species is an induced ovulator [9], there was rarely an opportunity for uterine tissue to be influenced by endogenous progesterone unless following natural corpus luteum formation after mating and ovulation opportunities. An alternative explanation for observations made in the present study was provocation of hyperplasia and other severe pathologies from prolonged reproductive cyclicity in the absence of occasional 'protective' progesterone afforded by ovulation and pregnancy [31]. For example, cheetah females in nature produce a first litter at ~3 y of age and then subsequent litters every 2 y until death [2, 7]. As long as competent males are in the vicinity, wild cheetah females appear to experience little time cycling and rather are either pregnant, lactating or raising young [7, 8]. Therefore, a major difference between the uterus of cheetahs living in situ versus ex situ is that the former are more exposed to endogenous progesterone whereas the latter are experiencing waves of estrogen (associated with frequent cyclicity) punctuated by bouts of ovarian quiescence [9]. For cheetahs managed in captivity that fail to mate and ovulate, this non-progestogenic environment could continue for more than 8 y. The dominant presence of estrogen then may contribute to a proliferative impact on endothelial tissues, with one mechanism of action perhaps expressed via an epidermal growth factor (EGF) pathway [59, 60]. In the uterus, estradiol induces the EGF receptor to stimulate cellular replication [59], and, specifically, both in vivo (rat) and in cell cultures (human), estrogen up-regulates uterine EGF receptor numbers and, therefore, overall cellular growth and proliferation of the endometrium [60, 61].

Regardless of the causative source and mechanism of action, the direct impact of aging on increasing insidious pathologies within the uterus likely influences the capacity to produce offspring, even in well-timed ovulating and successfully mated or artificially inseminated females. Presuming that there is a protective effect of progesterone in felids, then there also are practical implications, perhaps even offering suggestions for how to reduce the risk of endometrial hyperplasia and associated lesions. Munson et al. [31] have predicted that, compared to multiparity, a nulliparous condition increases the chances for a compromised uterus and, thus, infertility in felids. Our dataset on uterine histology was unhelpful in supporting this claim, largely because only a few of the animals in the young (n = 1) and prime (n = 5) age groups had previously produced young. Nonetheless, if periodic progesterone exposure is safeguarding to the felid uterus, it does not protect long-term. Within our histological dataset, >40% (n = 32) of the older group was multiparous, yet 87.5% of these (n = 28) had endometrial hyperplasia and ~44% of these (n = 14) experienced other serious uterine pathologies, including pyometra, adenomyosis and endometritis. Yet, it is possible that duration from last exposure to steroid is important. This might explain our discovery that cheetahs were more likely to experience uterine hyperplasia as the interval between last litter and death increased. Nonetheless, other factors are known to predispose a carnivore to uterine hyperplasia and associated pathologies. For example, in the cat, elevated serum estradiol levels [30], exposure to progestins, advancing age as well as nulliparity [31] predispose individuals to hyperplasia. Similarly, hyperplasia in canine species is believed to largely be related to exposure to progestins, advancing age [62], and nulliparity [46], with diestrus and postpartum metritis also leading bitches to hyperplastic conditions [46]. Regardless, our observations provide justification for the need for more detailed explorations of steroidal influences on felid uterine integrity. Meanwhile, these findings supported the practical notion that more effort be made to achieve pregnancies in young cheetahs managed in security populations, including achieving all needed reproduction in early years (<8). As we discovered severe uterine pathologies even in individuals considered of 'prime' age, it appears prudent to focus on reproducing cheetahs during the first 5 y of life, similar to what occurs in nature [7, 8].

Beyond our primary focus of examining the influence of animal age on the cheetah's ovary, oocyte and uterine integrity, it was encouraging to determine the possibility of consistently producing embryos in vitro. Embryo technologies, including IVF and embryo transfer, are not routinely used in the ex situ management of genetically valuable, rare felid species [4, 50, 63]. Nonetheless, there are important examples of how these collective approaches have been used to produce 'milestone' births, including in the African wild cat, caracal [64, 65] and ocelot [63]. Therefore, the biological potential of felid embryos generated from IVM/IVF has been demonstrated, suggesting the potential of this approach for ramping up animal numbers and ensuring the retention of genetic diversity in intensively managed populations. In vitro fertilization can be challenging in felids, including the cheetah, often related to a preponderance of pleiomorphic spermatozoa in the ejaculate [11, 41, 42, 66]. In the only earlier publication involving gamete interaction in culture for the cheetah [14], 12 females were treated with eCG/hCG and produced an average of 24 mature follicles and 23 oocytes recovered per animal. Approximately 90% of these were classified as mature (based on gross morphology) and 26% demonstrated evidence of fertilization with 11 embryos developing to the 16-cell stage and none beyond. By contrast in the present study, we recovered an average of 15 oocytes per female (across a diverse age group) with ~50% achieving maturation, 68% of mature oocytes fertilizing and 27 embryos reaching at least 16 cells in culture. The two studies differed vastly in how oocyte quality was assessed. In the earlier investigation, oocytes were determined to be mature on the basis of a simple visual assessment, specifically examining for expansion of cumulus oophorus cells [14]. It is likely that some, or even many, of the oocytes incubated with sperm in that study actually were immature, resulting in low fertilization and poor embryo development. Our contemporary investigation is strengthened by the ability to retrospectively evaluate nuclear maturation for all oocytes failing to cleave by 32 hpi using the advanced methods of microtubule immunostaining or Hoechst staining, thereby allowing nuclear status assessments of every aspirated oocyte. The current study also benefited from using high quality cryopreserved sperm samples from a limited number of donors. Fertilization was achieved by all samples from each male, with no influence of donor on subsequent embryo development.

Perhaps most important was our finding that there were no discernible differences in oocyte quality, fertilizability and early embryogenesis among the three cheetah age groups in the present study. Additional metrics such as assessment of embryo metabolism or live offspring production post-transfer would provide even greater insight into oocyte quality and developmental quality in this species. However, we find it encouraging that we were able to recover an average of nine oocytes from every cheetah in the 9 to 15 y age group of which almost seven met highest quality criteria. That the ability to achieve nuclear maturation and fertilization was no different than in the young and prime age groups also indicated that older females could produce significant numbers of embryos in vitro, including advancing to the morula stage (in at least 4 of the 9 older females). While the capacity of these embryos to translate into living offspring remains to be determined, the availability of viable-appearing oocytes from older females that mature, interact with sperm and form early blastocyst-stage embryos presents interesting opportunities for management and conservation. For example, most cheetahs >8 y of age now are disregarded as breeding candidates due to low fertility expectations. However, more than 35% of cheetahs in the contemporary North American population are in this age frame, with 85% of these having never reproduced. Our findings here suggest the need for a more serious examination of the potential role of embryo technologies in cheetah management -- specifically oocyte recovery and IVF followed by embryo transfer to already well-represented (and still young) cheetah recipients. It was noteworthy that embryo production in the present investigation was accomplished using conspecific spermatozoa cryopreserved and thawed using data from recent cryo-studies [42, 43]. Therefore, the source of male germplasm for future IVF efforts could be sperm from systematic sperm banking, including from free-living males [50]. Now that it is apparent that viable oocytes are

recoverable from even aged conspecifics, a logical next step is exploring IVF/embryo transfer as a way of avoiding loss of gene diversity, including from older females that likely will never reproduce by natural means.

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Figure Legends

Fig. 1. Cheetah ovaries and oocyte quality post-gonadotropin stimulation (eCG/hCG) and laparoscopic recovery. Ovary with (A) no follicular vascularization (score 0) versus (B) high degree of vascularization (score 3). Grade 1 (excellent) oocytes (arrow) (C) with uniformly dark cytoplasm and multiple layers of expanded cumulus cells. Oocytes: in normal anaphase I (D); with normal telophase spindle (green) and chromosome (white arrow) (E); with normal metaphase spindle (green) and chromosome plate (arrow) (F).

Fig. 2. Ultrasound images of an adult cheetah uterus: (A) uterine cornu total width; and (B) uterine cornu wall thickness after gonadotropin stimulation (arrows in both panels indicate uterine cornua; cursors demarcate portion measured).

Fig. 3. Histological examination of uterine tissues at necropsy. Biopsies were hematoxylin-eosin stained and graded for severity of hyperplasia: (A) cross-section of a normal (grade 0) cheetah uterus with no hyperplastic changes; (B) cross-section of a cheetah uterus with mild (Grade 1) cystic endometrial hyperplasia and minimal-to-mild proliferative and/or cystic changes in glands or surface epithelium without an increase in overall endometrial height; (C) cross-section of a cheetah uterus with moderate (Grade 2) hyperplastic and/or cystic change with an increased endometrial thickness of ≤ 2 times normal; (D) cross-section of a cheetah uterus with severe (Grade 3) hyperplastic and/or cystic changes with increased endometrial thickness of >2 times normal. Arrows denote areas of adenomyosis. In all micrographs, the bar = 500 μm.

Fig. 4. Average concentrations of estradiol (A-E) and progestagen (F-J) metabolites for cheetah females: (A, F) 45 days prior to eCG/hCG stimulation; (B, G) the 5 day peri-eCG/hCG interval (Day 1

= eCG injection day – Day +5); (C, H) Days +6 – +16; (D, I) Days +17 – +26; and (E, J) +27 – + 36. Within hormone (estradiol or progestagens) and for all age groups combined, values with different superscripts differ ($P < 0.05$).

Fig. 5. Incidence of oocyte maturation for cheetahs across three age groups. Values are means ± SEM.

Fig. 6. Morula (A) and blastocyst (B) produced by IVF (light microscopy). Cheetah blastocyst (C) stained with Hoechst to assess blastomere number. In all micrographs, the bar = 50 μm.

Fig. 7. Proportion of voucher specimens indicative of uterine endometrial hyperplasia and the incidence of severe uterine pathologies across cheetahs of three age groups. Within category, values with different superscripts differ ($P < 0.05$).

Table 1. Fertilization and embryo development in cheetah oocytes in vitro (means ± SEM).

	Age Group		
	Young (2 – 5 y)	Prime (6 – 8 y)	Older (≥9 y)
No. females	17	8	9
Combined ovarian volume[†] (mm^3)	769.5 ± 76.7	763.5 ± 111.8	644.4 ± 105.4
Average follicular vascularization[*]	1.7 ± 0.2	1.7 ± 0.4	1.8 ± 0.3
Average follicle number	20.6 ± 1.6[a]	17.2 ± 2.3[a,b]	11.6 ± 2.2[b]
Total no. oocytes recovered	222	98	77
Average no. oocytes recovered	16.7 ± 1.7[a]	16.6 ± 2.5[a]	9.6 ± 2.4[b]
Average no. Grade 1 and 2 oocytes	11.0 ± 2.7	11.9 ± 2.8	6.9 ± 2.5
Total no. oocytes inseminated in vitro	152	80	50
Metaphase II oocytes fertilized (%)	68.3 ± 17.5	67.7 ± 17.2	69.2 ± 15.7
Fertilized oocytes cleaved (%)	68.4 ± 22.4	53.6 ± 23.2	63.0 ± 19.9
Cleaved oocytes reaching 8 cells (%)	34.7 ± 13.6[a,b]	14.9 ± 14.1[b]	63.3 ± 12.0[a]
Cleaved oocytes reaching morula stage (%)	14.5 ± 11.1[a]	0 ± 0[a]	50.6 ± 9.8[b]

[a,b]Within rows, values with different superscripts differ ($P < 0.05$).
[†]Ovarian volume calculated as [(4 x π x 0.5 height x 0.5 length x 0.5 width)/3].
[*]Vascularization score of 0 – 3, with 0 = no vascularization and 3 = highly vascularized.

Table 2. Cheetah uterine metrics as assessed by ultrasonography of living individuals (means ± SEM).

	Age Group		
Metric	Young (2 – 5 y)	Prime (6 – 8 y)	Older (≥9 y)
No. females	17	8	9
Uterine body width (mm)	11.1 ± 1.4	12.9 ± 0.9	11.3 ± 0.8
Uterine body wall thickness (mm)	3.7 ± 0.5	4.4 ± 0.3	4.3 ± 0.3
Left cornu wall thickness (mm)	2.8 ± 0.3	2.8 ± 0.2	2.9 ± 0.2
Right cornu wall thickness (mm)	2.7 ± 0.3	2.9 ± 0.2	3.0 ± 0.2
Left uterine cornu width (mm)	7.0 ± 0.8	7.2 ± 0.6	7.2 ± 0.5
Right uterine cornu width (mm)	6.8 ± 0.6	7.3 ± 0.5	7.1 ± 0.4
Incidence of uterine cysts (%)	11.8	12.5	11.1
Incidence of hydrometra (%)	17.7	25.0	11.1

Fig. 1

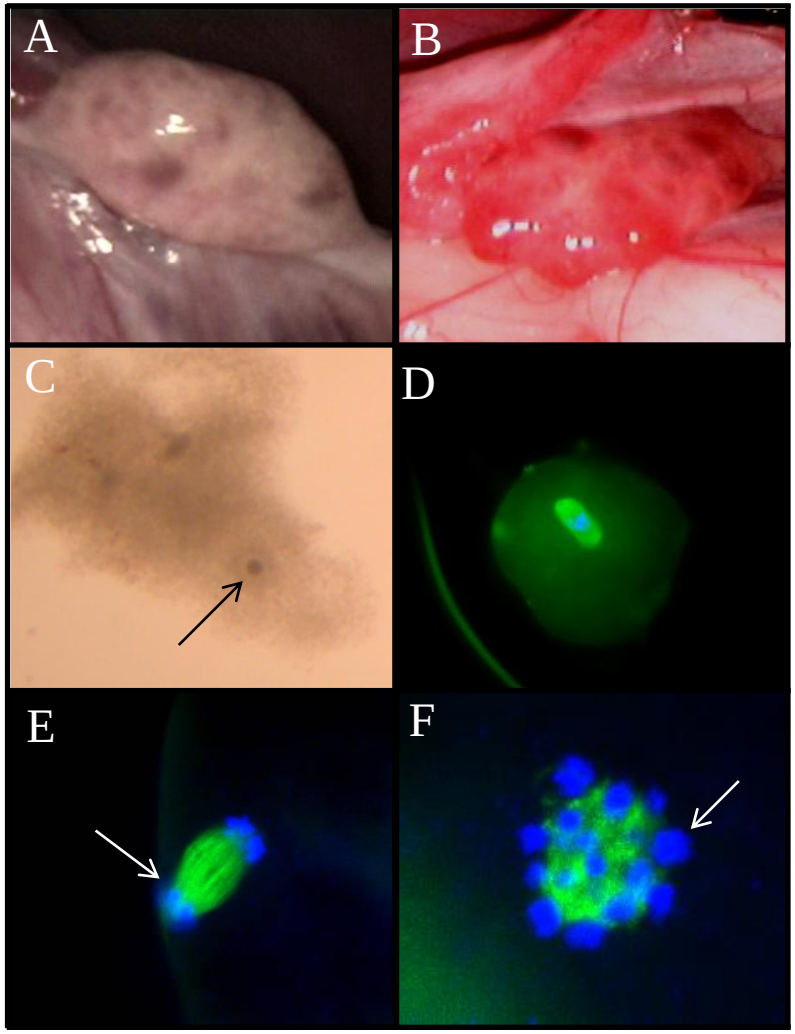


Fig. 2

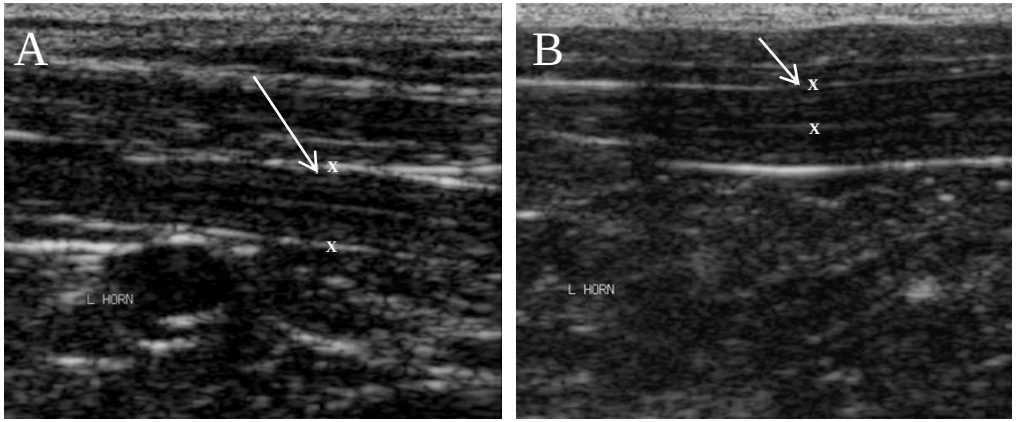


Fig. 3

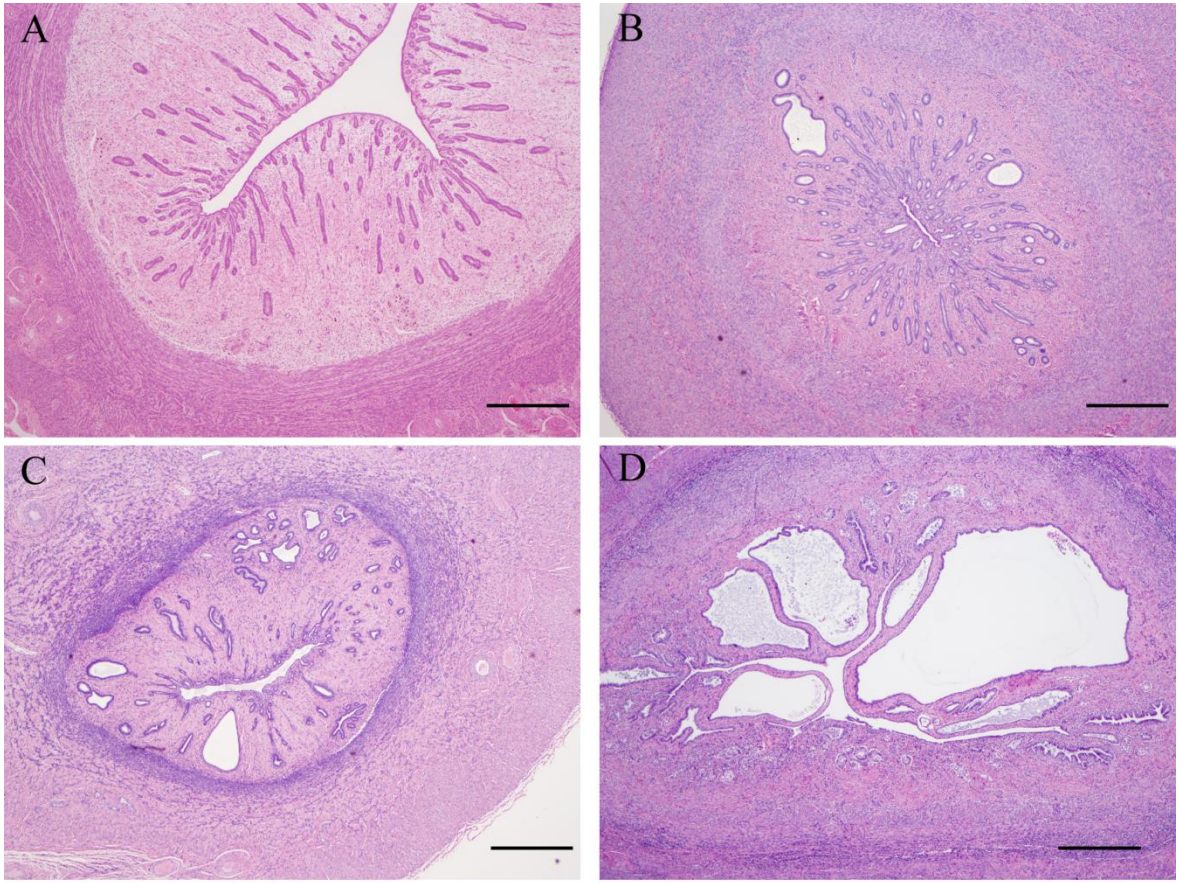


Fig. 4

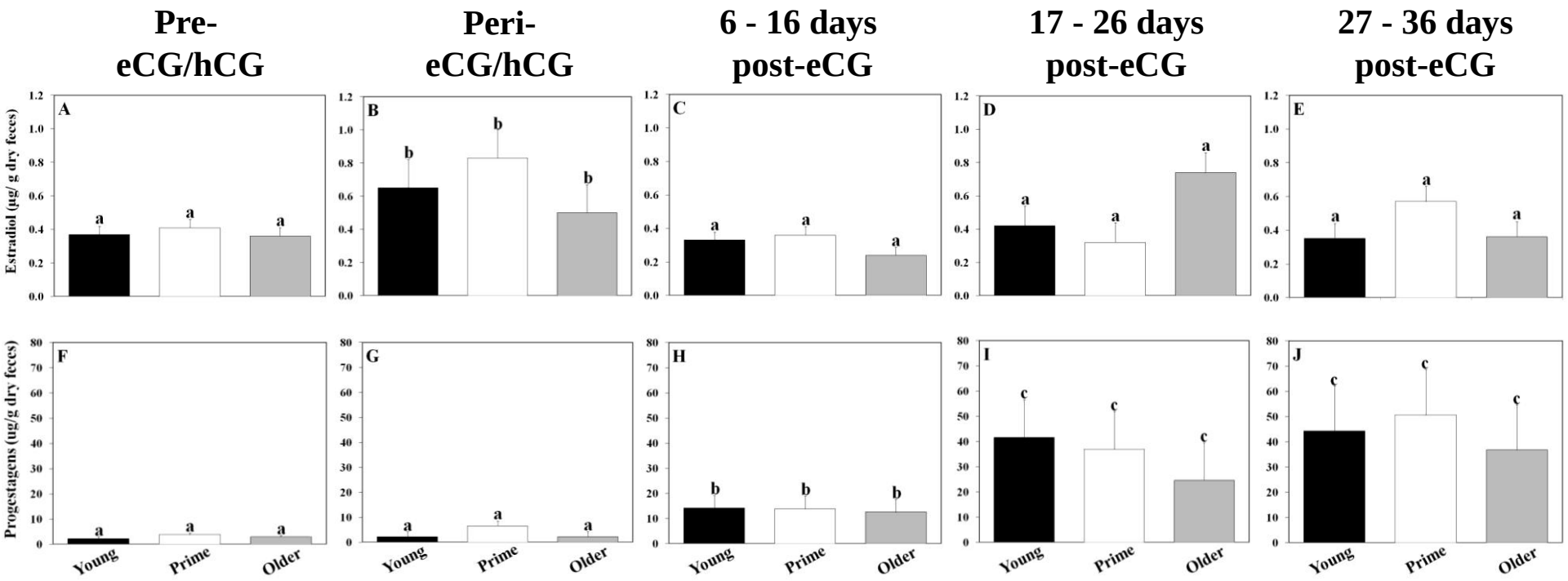

Pre-eCG/hCG
Peri-eCG/hCG
6 - 16 days post-eCG
17 - 26 days post-eCG
27 - 36 days post-eCG
Estradiol (µg/ g dry feces)
Progestagens (ug/g dry feces)
A
B
C
D
E
F
G
H
I
J
Young
Prime
Older

Fig. 5

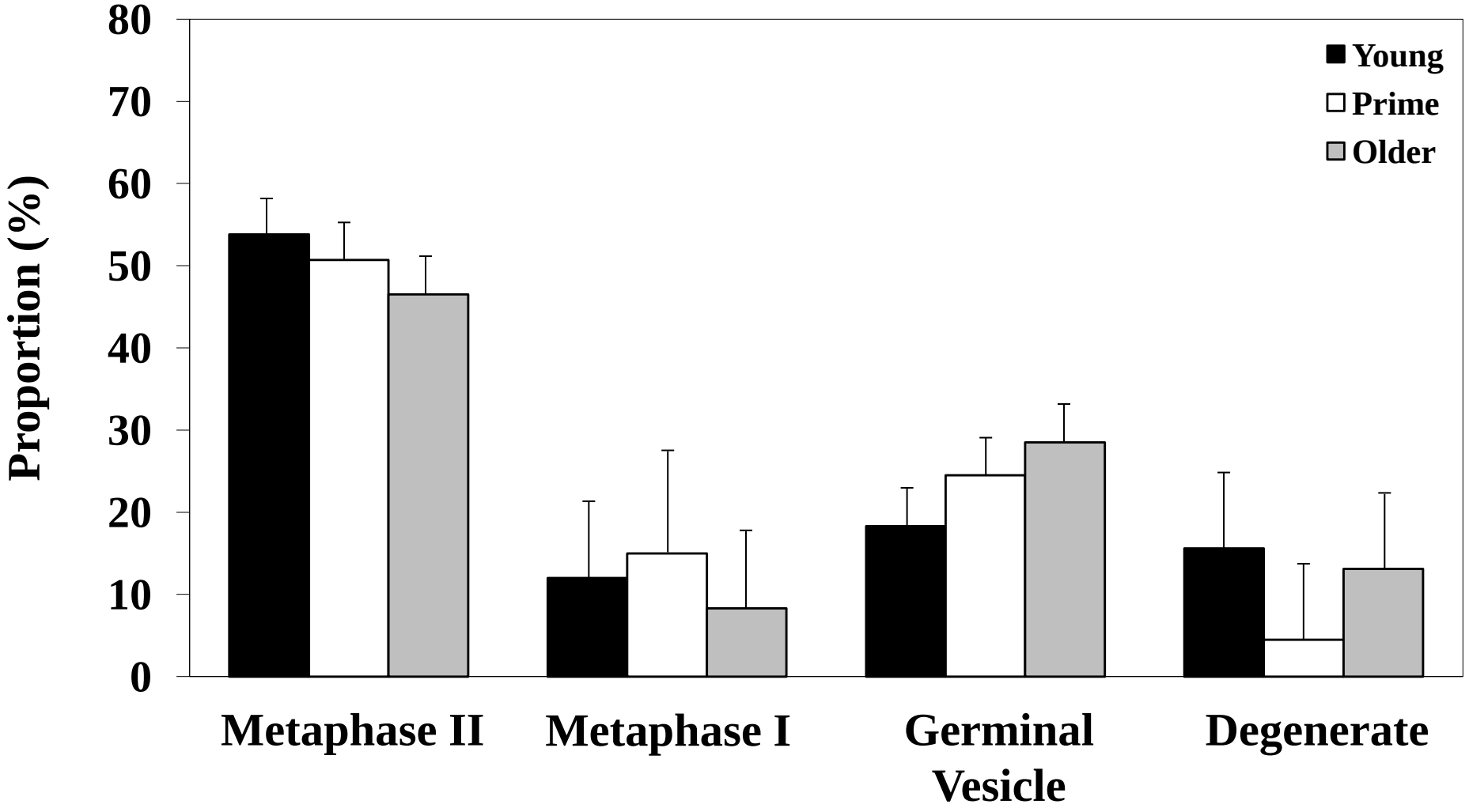

80
70
60
50
40
30
20
10
0
Proportion (%)
Young
Prime
Older
Metaphase II
Metaphase I
Germinal Vesicle
Degenerate

Fig. 6

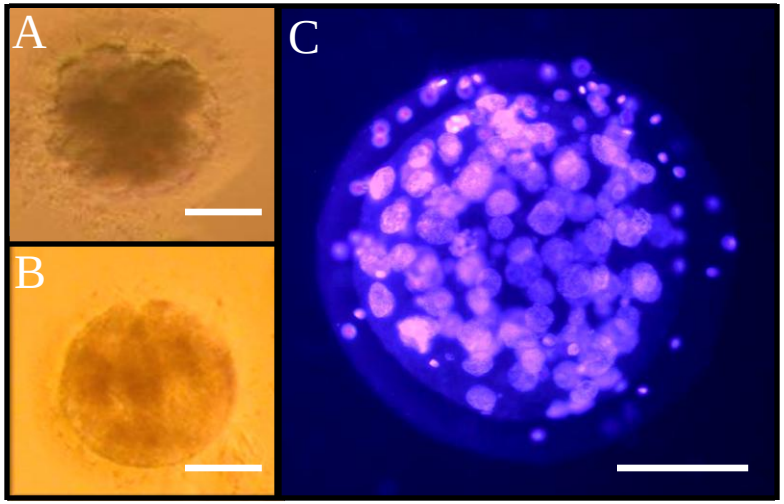
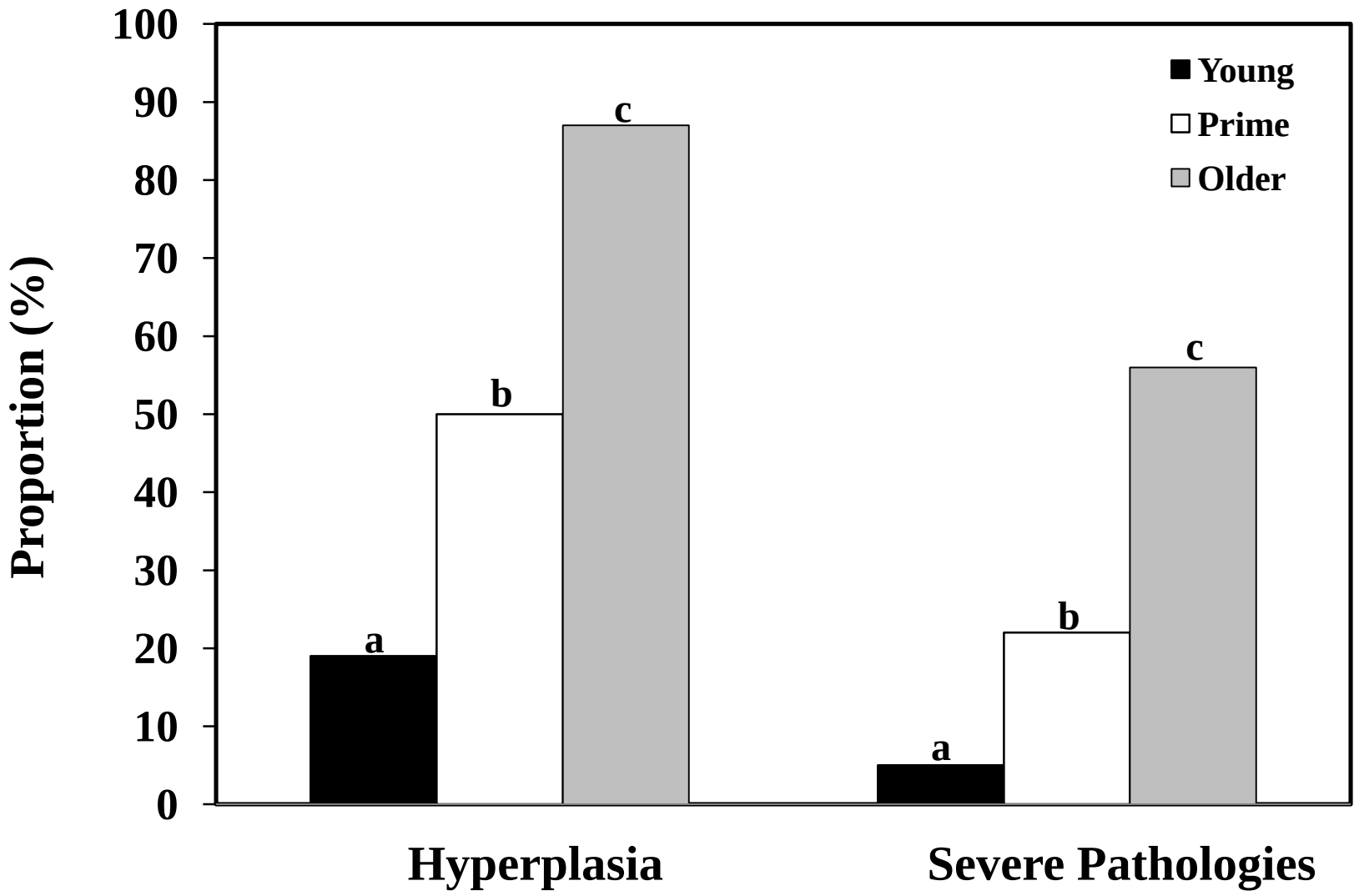


Fig. 7


100
90
80
70
60
50
40
30
20
10
0
Proportion (%)
Young
Prime
Older
a
b
c
a
b
c
Hyperplasia
Severe Pathologies