Received: 30 November 2023 | Accepted: 5 December 2023

DOI: 10.1002/jwmg.22541

RESEARCH ARTICLE

THE JOURNAL OF WILDLIFE MANAGEMENT | THE WILDLIFE SOCIETY

Effect of age, pregnancy, and tuberculosis status on oocyte parameters in African buffalo

Michal A. Kosior[1] | Kirsty Y. Lyne[2] | Francesca Salerno[1] | Bianca Gasparrini[1] | Giulia Esposito[2]

[1]Department of Veterinary Medicine and Animal Production, University of Naples Federico II, Naples 80100, Italy

[2]Department of Animal Sciences, Faculty of AgriScience, Stellenbosch University, Stellenbosch, Western Cape 7600, South Africa

Correspondence
Giulia Esposito, Department of Veterinary Science, University of Parma, Parma 43126, Italy.
Email: giulia.esposito@unipr.it

Present address
Giulia Esposito, Department of Veterinary Science, University of Parma, Parma 43126, Italy.

Funding information
South African Agency for Science and Technology Advancement, Grant/Award Number: RDYR160329161219–1617245

Abstract

In the past decades, the African buffalo (*Syncerus caffer*) population has steadily declined; an estimated reduction of 31.2% over 3 generations has resulted in classifying it as near threatened by the International Union for Conservation of Nature. Therefore, the aim of this work was a preliminary evaluation of the size and number of ovarian follicles and oocyte quality and quantity in African buffaloes in relationship to age, tuberculosis, and pregnancy status to assess feasibility of *in vitro* embryo production (IVEP) as a tool for conservation and propagation of this species. The study occurred during the winter dry season (Jul–Aug) of 2018 within Huluhluwe-Imfolozi National Park in Kwa-Zulu Natal, South Africa during the routine culling procedures of African buffalo carried out at the park by the provincial authorities as means of disease management and population control. We obtained ovaries from 39 adults and 10 juveniles, and transported them to the field laboratory where we counted follicles, collected oocytes by aspiration and slicing, and classified oocytes according to their morphology. Adult animals had more small and medium follicles compared to juveniles (small: 14.5 ± 0.1 [SE] vs. 14.0 ± 0.1; medium: 4.6 ± 0.7 vs. 2.0 ± 1.3); however, juvenile animals had more cumulus-oocyte complexes (COCs). Pregnancy and tuberculosis status did not affect COC recovery rate and quality. The oocyte recovery rate is comparable to cattle

and higher than in water buffalo (*Bubalus bubalis*). Therefore, our results suggest that IVEP could be an effective tool for conservation of valuable germplasm in African buffalo. The use of aspiration, using an 18-gauge needle and syringe, followed by slicing the ovary with a scalpel blade increased the recovery of suitable COCs (aspiration = 37.2 ± 3.02%; aspiration and slicing = 46.1 ± 3.02%), supporting the use of aspiration and slicing to optimize oocyte collection for conservation of African buffalo.

KEYWORDS
African buffalo, aspiration and slicing techniques, bovine tuberculosis, follicle IVEP, oocyte, pregnancy, *Syncerus caffer*

The African buffalo (*Syncerus caffer*) is a large, gregarious herbivore found throughout sub-Saharan Africa. Its population has steadily declined because of increased urbanization, agriculture, and the implementation of culling programs (Glanzmann 2016). The African buffalo is a major vector for the transmission of notable diseases such as bovine tuberculosis (BTB) and foot and mouth disease in South Africa, which have a large effect on livestock (Renwick 2007, Miller 2013). An estimated population decline of 31.2% over 3 generations has resulted in the species being listed by the International Union for Conservation of Nature as near threatened (International Union for Conservation of Nature 2019). Currently, there are no conservation programs in place for the protection of African buffalo (Bengis 1988, Campanile 2010).

Although *in situ* conservation could be a solution to this problem, the current disease status and a shortage of open space to maintain free-roaming populations without widespread poaching and hunting activities make *in situ* conservation programs problematic to be implemented (Wildt 2000). In such cases, *ex situ* conservation could be a powerful tool in maintaining species diversity and preventing the spread of disease (Wildt 1997, Leon-Quinto 2009). *Ex situ* conservation methods encompass a range of assisted reproductive technologies, such as artificial insemination and *in vitro* embryo production (IVEP), that can be used for maintaining species diversity and propagating valuable germplasm (Andrabi 2007, Leon-Quinto 2009). Although widely implemented in domestic animals and even humans, the use and success of these technologies have been limited in wildlife species primarily because of a lack of fundamental understanding of reproductive physiology and functioning (Schmidt 2006, Andrabi 2007). The use of *ex situ* technologies in the African buffalo has been limited and success rates have been poor, likely because most methodologies employed have originated from procedures implemented in domestic bovine species and have not been specific to the African buffalo (Shaw 1995, Kidson 1998). Although there are many phenotypic similarities between these closely related species, there can be differences in the reproductive physiology and anatomy between them (Schmidt 2006). Studies focusing on gross anatomy and physiology have a stronger relationship between a variety of buffalo species of the Bovidae family in contrast to the relationship between buffalo and cattle (Schmidt 2006). Assisted reproductive techniques such as artificial insemination and IVEP have been used successfully in cattle and water buffalo (*Bubalus bubalis*) for many years, with great benefits to breeding programs (Baruselli 2018, Ferré 2020). The embryo cryopreservation allows the movement of genetic material all over the world without the need to move animals, which is useful when breeding dangerous and high-value species, such as the African buffalo. Oocytes can even be collected and stored from animals that are deceased (Das 1996, Andrabi 2007). The possibility of using oocytes from deceased animals make this technique even more appealing in wildlife, where the access to biological material is often scarce. The use of IVEP in wildlife and, specifically, in African buffaloes, would allow biologists to overcome difficulties such as reproductive seasonality and poor estrus detection, which limit the success of other breeding techniques such as artificial insemination.

Another advantage of using IVEP is the possibility of producing more embryos at one time from the same donor and using different recipients for the transfer, thus speeding up genetic improvement and repopulation. Despite the great improvement in IVEP efficiency in domesticated livestock over the past years, this has not been the case for the African buffalo for which the success rate still lies at about 2% of embryo development because the process often stalls at the morula stage (Lambrechts 1999, Purohit 2003, Herold 2004). This difference in success rates among species is likely induced by several factors such oocyte quality, follicular environment, fertilization, and embryo culture environment. Furthermore, a main limitation to embryonic production in the domestic water buffalo, the morphologically most similar species to the African buffalo, is a scarce follicular population, resulting in a poor number of recruitable oocytes (Manik 2002).

Our objective was to conduct a preliminary evaluation of the follicle and oocyte population in female African buffalo of different ages and under different conditions (BTB and pregnancy status) culled during the winter dry season (Jul–Aug) in 2018 in the Huluhluwe-Imfolozi National Park in Kwa-Zulu Natal, South Africa, to gather information on the feasibility of the IVEP technique. Thus, the objectives were to analyze the size and number of ovarian follicles and oocyte quality and quantity according to age, pregnancy, and BTB status. Furthermore, we evaluated the oocyte recovery rate using 2 different techniques (aspiration and aspiration plus slicing) to determine the best approach to maximize the use of biological material.

STUDY AREA

We conducted this study within Huluhluwe-Imfolozi National Park in Kwa-Zulu Natal, South Africa (28.2198° S, 31.9519° E). The landscape is undulating to hilly, with a gradual drop in altitude from 580 m to 90 m from west to east along the Natal Monocline and occupies an area of 960 km^2. We conducted the study during the winter dry season (Jul–Aug) in 2018. In this period the average rainfall was 38 mm and the temperature ranged from 24–22°C during the day and 17–14° during the night. The vegetation in the dry season was low and the animals tended to gather more at the water sources. The park is home to 86 species of animals including the African buffalo, elephant (*Loxodonta africana*), black rhino (*Diceros bicornis minor*), white rhino (*Ceratotherium simum*), lion (*Panthera leo melanochaita*), leopard (*Panthera pardus pardus*), Nile crocodile (*Crocodylus niloticus*), hippopotamus (*Hippopotamus amphibius*), spotted hyena (*Crocuta crocuta*), giraffe (*Giraffa giraffa*), zebra (*Equus quagga burchellii*), nyala (*Tragelaphus angasii*), kudu (*Tragelaphus strepsiceros*), impala (*Aepyceros melampus*), blue duiker (*Philantomba monticola*), warthog (*Phacochoerus africanus*), bushpig (*Potamochoerus larvatus*), black baboon (*Papio ursinus*), numerous species of amphibians and reptiles, and about 340 species of birds.

METHODS

The provincial authorities collected samples and data within the routine culling procedures of African buffalo as a means of disease management and population control. For this purpose, they mass-captured the animals via helicopter and herded them towards transportable bomas (enclosing structures for wildlife or livestock animals) that were set up in the field to contain the buffalo herds (Kock 2012) where the provincial authorities divided the herd into smaller groups of 15–20 animals using gangways and holding pens for immobilization and testing. Prior to handling, the responsible veterinarian immobilized all animals using a mixture of the opioid derivative etorphine hydrochloride (M99, Novartis Animal Health, Isando, South Africa) and the butyrophenone tranquilizer azaperone (Stresnil, Janssen Pharmaceutica, Woodmead, South Africa), with various combinations of dosages depending on age of animals: 4–6 mg M99 and 60–100 mg azaperone for young animals (calves and juveniles; Table 1), and 8–10 mg M99 and 150–200 mg of azaperone cocktail for young adult and adult African buffaloes. Crews marked animals with an ear tag (Z EconoTags, ZeeTags, North Harbour, Auckland, New Zealand) placed on the left ear and positioned between the

TABLE 1 Characteristics used to determination age of African buffalo females, according to Taylor (1988), for animals culled during the winter dry season (Jul–Aug) in 2018 in the Hluhluwe-Imfolozi National Park in Kwa-Zulu Natal, South Africa.

Age category	Dentition	Horn size
Calf (0–2 years)	0 front teeth	Small stubs
Juvenile (2–3 years)	2–4 permanent front teeth	Small curve in horns
Young adult (4–5 years)	6 permanent front teeth	Bony ridge over the center of the head
Adult (>5 years)	8 permanent front teeth	Fully developed horns

ear veins, in the central part of the ear, and brands with a unique and unambiguous number. They tested buffaloes for BTB via the single intradermal comparative cervical tuberculin test and blood serum-based antibody test (IDEXX, Westbrook, ME, USA) to test for *Mycobacterium bovis*, as previously reported (van der Heijden et al. 2016). After application of the skin test and sampling, the immobilization drugs were reversed using diprenorphine hydrochloride (M5050, Novartis Animal Health) at twice the dose of M99. We identified animals by sex and estimated age by development of the teeth, horns, and body size according to Taylor (1988; Table 1).

When >60% of captured animals were positive for BTB, the entire herd was culled to prevent further spillover of infections. Animals were culled via rifle shot to the head. Crews drained the culled animals of blood in the field within half an hour of death. They then transported carcasses to the internal abattoir for further processing within 2 hours of exsanguination.

Processors collected the reproductive tracts of 52 female African buffaloes during the evisceration process. They removed all reproductive organs from the carcass and recorded data pertaining to each animal such as presumptive age, BTB status, and pregnancy status. Ovaries were dissected free from their connective tissue and transported in physiological saline solution at 33–35°C to the laboratory for further processing within 4 hours from culling. We recorded the number of visible follicles, size of follicles, and the presence of a corpus luteum. We measured the size of visible follicles using a digital caliper. According to the classification of Abdoon and Kandil (2001), we grouped visible follicles based on their diameter into small (<3 mm), medium (>3 mm to <9 mm), and large (>10 mm).

We then aspirated visible follicles with an 18-gauge needle and syringe using tissue culture medium-hepes (TCM-hepes; Gibco, Life Technology, Paosley, United Kingdom) supplemented with 3% fetal calf serum (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) and 50% heparin at a concentration of 522 U/ml (Sigma Aldrich). We deposited aspirated fluid from each ovary into a 65-mm cell culture petri dish. Under a stereomicroscope (Nikon SMZ800; Nikon, Tokyo, Japan) with a magnification of X20, we identified cumulus-oocyte complexes (COCs) and collected and morphologically graded ovaries based on the characteristics of the cumulus cells and cytoplasm as previously described (Di Francesco 2012) with minor modifications. Briefly, we classified the oocytes as grade A if oocytes had >3 layers of cumulus cells and homogenous cytoplasm (Figure 1), grade B if oocytes had ≥2 layers of cumulus cells and homogenous cytoplasm, grade C if oocytes were partially denuded but still showed homogenous cytoplasm, grade D if oocytes were totally denuded but still showed homogenous cytoplasm (Figure 1), grade E if oocytes had expanded cumulus and homogenous cytoplasm (Figure 1), grade F if oocytes were degenerated with irregular shrunken cytoplasm, and grade ZP (zona pellucida) if oocytes were totally denuded with no visible cytoplasm. We calculated the recovery rate of COCs as the number of COCs collected via aspiration compared to the number of counted ovarian follicles. For each animal we recorded the recovery rate, the number of total COCs, COCs suitable for IVEP (grade A + B + C), and total discarded COCs (D + E + ZP).

After aspiration of follicles, we sliced and minced ovaries with a number 10 scalpel blade and rinsed them in a 65-mm petri dish with TCM-hepes supplemented with 3% fetal calf serum (Sigma Aldrich) and 50% heparin at a concentration of 522 U/ml (Sigma Aldrich). We examined the petri dishes under a stereomicroscope (Nikon SMZ800) and collected COCs of all qualities. We recorded the number and quality of COCs recovered during the

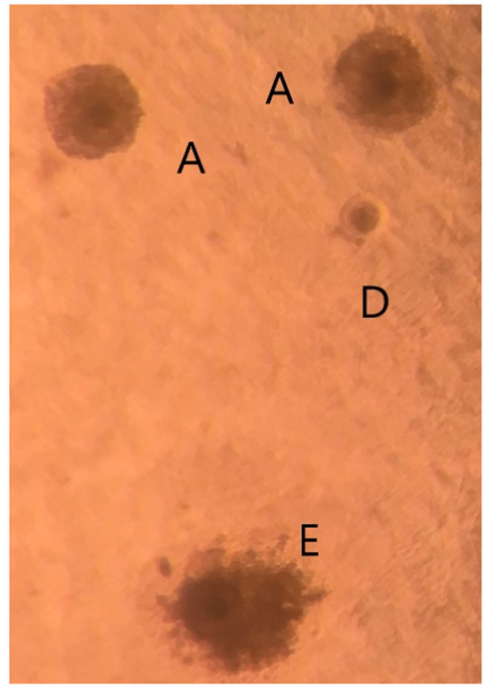

FIGURE 1 Oocytes of grade A, D, and E observed under a stereomicroscope at a magnification of ×20 harvested from an African buffalo culled during the winter dry period (Jul–Aug) in 2018, within Huluhluwe-Imfolozi National Park in Kwa-Zulu Natal, South Africa. Grade A oocytes have more than 3 layers of cumulus cells and homogenous cytoplasm; grade D oocytes are totally denuded oocytes but still showing homogenous cytoplasm; and grade E oocytes present an expanded cumulus and homogenous cytoplasm.

slicing process. We excluded oocytes with a small diameter (<100 µm; Hyttel 1997), likely collected from preantral follicles, from the analysis.

Among the 52 female African buffaloes, 39 were classified as adults, 10 as juveniles, and 3 as young adults; we excluded the 3 young adults from analyses. We conducted descriptive analyses of the follicles and oocyte quality and quantity using the univariate procedure (SAS 9.4, SAS Institute, Cary, NC, USA). We evaluated follicle size and quantity, and oocyte quality and quantity using analysis of variance for repeated measures with the GLIMMIX procedure (SAS 9.4). We analyzed data from each ovary (right and left) by size of the ovary, pregnancy status, age, BTB status, and their interactions. All pregnant animals were in the adult age group; hence, we did not analyze data by age.

RESULTS

We obtained samples from 52 females and included 39 adults and 10 juveniles in this analysis. We did not observe differences in the mean number of follicles between adult and juvenile buffaloes; however, the number of small and medium follicles was higher in adults than in juveniles ($P \leq 0.001$). The mean number of follicles was similar between right and left ovaries in both age categories of animals for small, medium, and large follicles. In juvenile females, the number of large follicles in the left ovary was greater than in the right ovary ($P \leq 0.05$; Table 2).

The mean number of oocytes recovered per animals was of 18 ± 12.5 (SD) in juveniles and 11.9 ± 7.8 in adult buffaloes. Among these, the COCs suitable for IVEP were 8.2 ± 5.1 for adult buffaloes and 9.7 ± 7.4 for juveniles

from which 3.1 ± 2.4 were COCs of grade C; only 2.4 ± 2.7 COCs of grade C were recovered from the adults. The analysis of variance confirmed a higher recovery rate of COCs after aspiration in the juvenile animals compared to the adults ($P \leq 0.05$); however, we did not observe differences between groups for suitable COCs (Table 3). We did not find evidence for interactions between variables.

Among the 39 adult females, 20 were pregnant with 11 carrying their pregnancy in the left horn and 9 in the right horn of the uterus. The number of follicles was higher ($P \leq 0.05$) in the non-pregnant buffalos compared to

TABLE 2 Population of total follicles and of different size follicles (small, medium, large) on the ovaries (right, left) of juvenile ($n = 10$) and adult ($n = 39$) female African buffaloes culled during the winter dry period (Jul–Aug) 2018 within Huluhluwe-Imfolozi National Park in Kwa-Zulu Natal, South Africa. Data are expressed as least squares mean (±SE).

	Right ovary		Left ovary		Total	
Follicles	**Adult**	**Juvenile**	**Adult**	**Juvenile**	**Adult**	**Juvenile**
Small	7.9 ± 1.2	6.3 ± 1.2	6.6 ± 1.0	7.6 ± 2.0	14.5 ± 0.1**	14.0 ± 0.1**
(%)[a]	69.3 ± 13.0	66.4 ± 13.2	63.2 ± 30.9	80.3 ± 16.8	67.6 ± 4.9	71.1 ± 9.5
Medium	2.1 ± 0.4	0.8 ± 0.7	2.3 ± 0.4	1.2 ± 08	4.6 ± 0.7*	2.0 ± 1.3*
(%)[a]	17.6 ± 2.9	14.8 ± 5.4	24.4 ± 4.5	13.1 ± 8.8	20.6 ± 3.2	14.1 ± 6.2
Large	0.9 ± 0.3	1.1 ± 0.6	1.0 ± 0.3	0.6 ± 0.5	1.9 ± 0.4	1.7 ± 0.9
(%)[a]	13.1 ± 4.3	18.8 ± 8.4	10.9 ± 3.4	8.2 ± 6.6	11.4 ± 3.1	14.2 ± 6.0
Total	10.9 ± 1.2	8.2 ± 2.3*	10.3 ± 1.2	9.5 ± 3.1*	21.2 ± 1.9	18.9 ± 3.8
(%)[b]	50.8 ± 6.2	46.5 ± 24.7	49.2 ± 2.9	53.5 ± 5.5		

[a]Percentage in each size category over total follicles on the right or left ovary.

[b]Percentage of total follicles on the right or left ovary per animal.

*Values differ between adults and juveniles or right and left ovaries ($P \leq 0.05$).

**Values differ between adults and juveniles ($P \leq 0.01$).

TABLE 3 Oocyte quality in relation to age. Least squares means (±SE) of cumulus-oocyte complexes (COCs) of different quality in adult ($n = 39$) and juvenile ($n = 10$) female African buffaloes culled during the winter dry period (Jul–Aug) in 2018 within Huluhluwe-Imfolozi National Park in Kwa-Zulu Natal, South Africa. We grouped quality COCs (grades A, B, and C) and discarded COCs (grades D, E, and F).

	Right ovary		Left ovary		Total	
COCs	**Adult**	**Juvenile**	**Adult**	**Juvenile**	**Adult**	**Juvenile**
A + B + C	3.5 ± 0.5	4.4 ± 1.0	4.5 ± 0.6	4.9 ± 1.2	8.0 ± 0.1	9.3 ± 1.9
(%)[a]	22.4 ± 2.9	23.4 ± 5.7	32.9 ± 2.3	29.0 ± 3.4	55.3 ± 4.2	52.4 ± 8.1
Discarded	3.3 ± 0.6	3.5 ± 1.2	3.0 ± 0.6*	5.7 ± 1.2*	6.3 ± 1.1	9.2 ± 2.1
(%)[a]	22.9 ± 3.3	14.9 ± 6.4	21.8 ± 2.6*	32.8 ± 5.1*	44.7 ± 4.2	9.2 ± 2.1
Total	6.9 ± 1.0	7.9 ± 2.0	7.5 ± 1.1	10.5 ± 2.1	14.3 ± 1.4**	18.4 ± 6.0**
(%)[b]	30.3 ± 4.3	40.6 ± 6.3	42.9 ± 3.8**	46.7 ± 5.8**	66.1 ± 4.8*	80.0 ± 9.3*

[a]Percentage in each quality category over total aspirated COCs.

[b]COC recovery rate (percentage over total follicles).

*Values differ between adults and juveniles ($P \leq 0.05$).

**Values differ between adults and juveniles ($P \leq 0.01$).

pregnant adult animals. We did not observe differences in the percentage of medium and large follicles between groups, whereas the percentage of small follicles was higher in non-pregnant females ($P \leq 0.05$; Table 4). The higher number of follicles in non-pregnant females did not result in more total recovered COCs compared to the COCs recovered in pregnant animals (Table 5). Furthermore, the number of suitable COCs (grade A, B, and C) did not differ between groups (Table 5). On the other hand, when comparing the horns (pregnant and non-pregnant uterine horn) of pregnant females, we observed a trend for a higher number of suitable COCs in the pregnant horn compared to the non-pregnant one (least squares mean [LSM] ± SE: 4.0 ± 0.8 vs. 5.6 ± 0.8 for the non-pregnant and pregnant horn, respectively; $P = 0.057$). This trend is explained by the higher number of COCs of grade B in the pregnant horn (LSM ± SE: 2.0 ± 0.5 vs. 3.6 ± 0.5 for the non-pregnant and pregnant horn, respectively; $P = 0.003$).

We evaluated 40 BTB-positive and 12 BTB-negative animals in this study.

We did not observe differences in the mean number of follicles between BTB-positive (9.9 ± 1.3 and 9.8 ± 1.4 for right and left ovaries, respectively) and BTB-negative (9.3 ± 2.1 and 10 ± 1.9, respectively, for the right and left ovaries) buffaloes. Disease status of the animals and the interaction with age and pregnancy status had no effect on the number of visible follicles per ovary. When evaluating the number of follicles per donor, however, the BTB-positive females had a higher number of small follicles compared to the BTB-negative buffaloes (15.2 ± 0.3 vs. 13.2 ± 0.4; $P \leq 0.01$). When evaluating the COC recovery rate and quality per donor, there was no difference between groups in terms of total COCs and quality.

On average we collected 15.7 oocytes per animal regardless of age or pregnancy status using aspiration techniques alone, giving a mean recovery rate of 68.3 ± 26.2%. Slicing after aspiration resulted in an additional recovery of 3.3 COCs per animal. Thus, the mean recovery rate increased to 85.2 ± 32.4% through the slicing method after aspiration. The combined techniques (aspiration and slicing) resulted in a higher recovery rate of suitable COCs (37.2% ± 3.02 vs. 46.1% ± 3.02 with aspiration only and aspiration and slicing respectively; $P \leq 0.001$). Therefore, the slicing technique increased a recovery rate of suitable COCs of approximately 9%. We did not observe differences between age, BTB status, or pregnancy status for total COCs or quality of the oocytes recovered through slicing.

TABLE 4 Least squares means (±SE) of total small, medium, and large follicles, counted on the ovaries of pregnant and non-pregnant African buffaloes culled during the winter dry period (Jul–Aug) in 2018 within Huluhluwe-Imfolozi National Park in Kwa-Zulu Natal, South Africa.

	Right ovary		Left ovary		Total	
Follicles	Non-pregnant	Pregnant	Non-pregnant	Pregnant	Non-pregnant	Pregnant
Small	8.3 ± 1.3	5.9 ± 2.0	7.8 ± 1.1	6.5 ± 1.7	16.2 ± 0.3**	12.4 ± 0.02**
(%)[a]	65.6 ± 14.5**	70.1 ± 14.5**	64.1 ± 16.3	79.4 ± 18.5	66.1 ± 5.4	72.6 ± 8.3
Medium	2.6 ± 0.4**	0.3 ± 0.6**	3.2 ± 0.8**	0.7 ± 0.7**	5.8 ± 0.8**	0.9 ± 1.2**
(%)[a]	17.6 ± 2.9	14.8 ± 5.4	24.4 ± 4.5	13.1 ± 8.8	20.6 ± 3.2	14.1 ± 6.2
Large	0.9 ± 0.3	1.2 ± 0.5	1.0 ± 0.3	0.6 ± 0.4	1.9 ± 0.5	1.8 ± 0.7
(%)[a]	9.8 ± 4.8	22.1 ± 8.3	9.9 ± 3.8	9.2 ± 5.8	9.8 ± 3.4	15.8 ± 5.2
Total	11.8 ± 1.3*	7.3 ± 2.0*	12.0 ± 1.5	7.8 ± 2.3	23.9 ± 2.1*	15.1 ± 3.3*
(%)[b]	49.3 ± 18.3	48.1 ± 19.3	50.7 ± 3.1	51.9 ± 4.8		

[a]Percentage in each size category over total follicles on right or left ovary.

[b]Percentage of total follicles on the right or left ovary per animal.

*Values differ between pregnant and non-pregnant animals ($P \leq 0.05$).

**Values differ between pregnant and non-pregnant animals ($P \leq 0.01$).

TABLE 5 Oocyte quality (mean ± SD) as indicated by cumulus-oocyte complexes (COCs) recorded for pregnant and non-pregnant adult female African buffalo culled during the winter dry period (Jul–Aug) in 2018 within Huluhluwe-Imfolozi National Park in Kwa-Zulu Natal, South Africa. We grouped quality COCs (grades A, B, and C) and discarded COCs (grades D, E, and F).

	Right ovary		Left ovary		Total	
COCs	Non-pregnant	Pregnant	Non-pregnant	Pregnant	Non-pregnant	Pregnant
A + B + C	3.6 ± 0.9	4.3 ± 0.6	4.6 ± 1.1	4.7 ± 0.7	8.3 ± 1.8	9.0 ± 1.1
(%)[a]	20.4 ± 5.1	25.3 ± 3.2	35.1 ± 2.3	26.8 ± 5.4	55.5 ± 7.3	52.2 ± 4.6
Discarded	3.2 ± 1.1	3.6 ± 1.0	4.4 ± 1.1	4.3 ± 0.7	7.6 ± 1.9	7.9 ± 1.2
(%)[a]	14.1 ± 5.8	23.7 ± 3.6	30.4 ± 4.6	24.1 ± 2.9	44.6 ± 7.3	47.8 ± 4.6
Total	6.8 ± 1.8	7.9 ± 1.1	9.0 ± 1.9	9.0 ± 1.2	15.8 ± 9.5	16.9 ± 2.8
(%)[b]	32.1 ± 12.2**	38.9 ± 22.9**	47.1 ± 6.0	42.2 ± 3.8	79.4 ± 10.3	81.0 ± 8.1

[a]Percentage in each quality category over total aspirated COCs.

[b]COC recovery rate (percentage over total follicles).

**Values with different superscript in a row differ significantly ($P \leq 0.01$).

DISCUSSION

The present study aimed at evaluating the follicular and oocyte population in African buffaloes of different ages and under different conditions (BTB and pregnancy status) to assess the feasibility of the IVEP technique for conservation programs. The number of follicles per ovary recorded in the African buffalo was higher compared to water buffalo and lower compared to cattle. In domestic water buffaloes, reports about the mean number of follicles on the ovarian surface are controversial, with values ranging from 3.4 ± 0.2 or 4.8 ± 0.3 (Abdoon 2001, Di Francesco 2012) to 10 per ovary (Das 1996). On the other hand, in cattle a mean number of follicles per animal of 22.66 ± 12.56 in juvenile females, and 18.03 ± 10.83 in adult cows has been reported (Katska 1984). Therefore, our results suggest that African buffalo has a follicular population similar to cattle and much higher than domestic water buffalo. The lower number of follicles observed by many authors in the domestic buffalo is in line with what has been observed in autochthonous bovine breeds, often showing lower follicular populations (Li 2007, Presicce 2020). The number of primordial and antral follicles in domestic buffalo is lower than in cattle (Manik 2002), resulting in poor oocyte recovery, which represents the main limitation to IVEP technology (Baruselli 2020). Our results suggest that antral follicles are higher than in the domestic buffalo.

Age had a strong effect on the number of small and medium size follicles, which were fewer in the juvenile females. Furthermore, juvenile animals also had a higher number of non-suitable oocytes. This is somewhat consistent with the observation that nulliparous Murrah buffalo (domestic water buffalo) oocytes have lower embryonic yields when compared with those from adult animals (de Carvalho 2019). Unfortunately, because of the limitations of the study, we could not record mass of animals. The number of COCs recovered seems to be more similar to the number observed in cattle than in water buffalo. The Italian Mediterranean buffalo (domestic water buffalo) has a slightly lower number of aspirated COCs recovered per ovary, ranging between 0.7 and 4.3 (Di Francesco 2011, 2012). Whereas in cattle, the number of normally aspirated oocytes per animal varies between 5 and 10 (Wang 2007) and appears more similar to that observed in this study. One of the major factors negatively influencing the success of IVEP in domestic buffalo is the limited biological material, but our results suggest that IVEP could be an effective tool for conservation and propagation of valuable germplasm in the African buffalo. The slight decline in COC numbers during aging has been seen in several related species including cattle and water buffalo (Katska 1984, Nava-Trujillo 2020). In cattle, the number of COCs declined from 9.88 ± 6.24 in juvenile animals to 8.37 ± 5.87 in adults (Kątska 1984). Kątska (1984) also reported the number of grade A COCs was

greater in juvenile animals compared to adults. In our study, we did not observe differences between the age groups considered, but the adult category was not further subdivided according to age. This means that we do not actually have precise information on the age or number of births of these animals; similarly, we do not have data on the survival of older animals in the reserve (elimination by predators or culling in tuberculosis or other disease eradication programs). Regardless the age, the mean number of good quality COCs (A + B + C) is higher than reported in the Italian Mediterranean buffalo where the mean number of good quality COCs per ovary ranges between 0.4 and 2.4 (Di Francesco 2011) and again more similar to bovine (Sianturi 2002). A study on Egyptian buffalo (domestic water buffalo) of different ages reported an average of 2.2 COCs recovered per ovary after aspiration (Singh 2001) with 71% considered good quality from a morphological point of view. In our study, despite a higher number of COCs collected, we observed a similar percentage (68.3 ± 26.2%) of good quality COCs in adult animals. Non-pregnant animals show a higher number of visible follicles compared to pregnant animals. Among pregnant animals, a greater quantity of follicles and oocytes was recovered from the contralateral ovary to the site of pregnancy; this is likely due to the presence of the corpus luteum, which occupies a large part of the ovary. A similar effect has been observed in the water buffalo, where the presence of the corpus luteum always affects the number of oocytes available after aspiration (Singh 2001). However, there was no evidence of a difference ($P = 0.198$) for the number of recovered COCs, in accordance with studies conducted in Egyptian buffaloes (Abdoon 2001). Regarding oocyte quality, although there was no evidence of a relationship, we observed more good quality oocytes in non-pregnant animals; however, the percentage of oocytes that from a morphological point of view are suitable for IVEP were greater in pregnant animals. This is consistent with observations in bovine; in cattle, ovaries with corpus luteum showed a greater number of good quality oocytes (Penitente-Filho 2015). A similar phenomenon was also observed in the buffalo; according to a study conducted in Egypt, ovaries of Egyptian buffaloes with a corpus luteum produced more good quality oocytes (Abdoon 2001), but pregnant and non-pregnant animals did not differ in good quality oocytes when the ovaries were analyzed in pairs (Abdoon 2001). Obviously, without an indication about blastocyst yield, it remains a suggestive and plausible hypothesis, but it is possible to assume that as in other species of ruminants, the oocytes of pregnant animals can somehow benefit from high levels of progesterone (Takuma 2010). This indicates that germinal material from both empty and pregnant animals may be useful also in African buffalo.

Disease status of the animals and the interaction with age and pregnancy status had no effect on the total number of visible follicles per ovary and on the COC recovery rate and quality. When evaluating the total number of follicles per donor, however, the BTB-positive females had a higher number of small follicles compared to the BTB-negative buffaloes. None of the animals had macroscopic lesions attributable to the disease at the level of the genital system. A recent study conducted in Holstein Friesian cross breed cattle farmed in Ethiopia analyzed the effect of the BTB status on some reproductive parameters and indicated that the hazard ratio associated with BTB-positive animals was <1 for all fertility parameters, suggesting that BTB status increases the time between events; however, the estimated effect was only significant for the calving to service time (Tschopp 2021). To the best of our knowledge, no studies investigating the role that BTB may have on reproductive performance in African buffaloes have been conducted. Thus, although rare, we cannot exclude that if this disease affects the female genital system, it can negatively affect reproductive performance. Moreover, although the health status does not seem to influence the number or the quality of collected oocytes, it cannot be excluded that the germinal material of positive animals may be a vehicle of disease because there are no studies in the African buffalo on the efficiency of the zona pellucida as an effective barrier in protecting the germ cell from pathogenic microorganisms. In a study conducted on follicular fluid, oocytes, and embryos produced from bovine donors infected with *Mycobacterium avium* ssp. *paratuberculosis* and showing clinical symptoms, a certain percentage tested positive (Bielanski 2016), but when samples were collected from animals without clinical manifestation, it was possible to obtain negative COCs (Kruip et al. 2003). In our opinion, the risk-benefit ratio must be carefully evaluated before considering the use of germline material from BTB-positive animals. Furthermore, compliance with standardized hygiene procedures for taking and treating the material at the slaughterhouse and in the laboratory can reduce this risk. Finally, further studies are

necessary to quantify the risk, the extent of the colonization of the follicular environment by mycobacteria, and the efficiency of the protection offered by the zona pellucida in the African buffalo.

Follicular aspiration resulted in a recovery rate of 68.3 ± 26.2% regardless of age, pregnancy, and BTB status. Furthermore, a 32.7% increase in oocyte yield was achieved when slicing was performed after aspiration, increasing the percentage of recovery considerably, consistently reaching around 90–95%. Studies in cattle have reported similar results with a much higher COC yield when slicing methods have been used (Wang 2007). The increased recovery rate obtained with the addition of the slicing technique resulted in an increased recovery rate of suitable COCs. The recovery rates described above demonstrate the advantages of implementing both aspiration and slicing methods for COCs recovery; therefore, we suggest the use of this technique to optimize the amount of material that can be obtained for conservation.

MANAGEMENT IMPLICATIONS

The results of our study suggest the feasibility of IVEP as a tool to rescue germinal material from dead African buffaloes. The use of germplasm from deceased animals could be the best option to minimize the handling of dangerous and high value animals when implementing IVEP programs. The results of the present study may also lay the groundwork for further research aimed to develop a species-specific IVEP system, and to study seasonal changes and hormonal profile relative to the stage of the cycle to define a successful IVEP program for the protection of the African buffalo.

ACKNOWLEDGMENTS

The authors acknowledge the staff at Huluhluwe-Imfolozi National Park in Kwa-Zulu Natal, South Africa, and in particular D. Cooper for his assistance in organizing the logistics.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENT

All experimental procedures were approved by the Animal Use and Care Committee of Stellenbosch University (project number ACU-2017-0477) and animals were handled in accordance with the South African National Council of Societies for the Prevention of Cruelty (NSPCA).

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ORCID

Giulia Esposito http://orcid.org/0000-0001-8386-1890

REFERENCES

Abdoon, A. S. S. 2001. Factors affecting number of surface ovarian follicles and oocytes yield and quality in Egyptian buffaloes. Reproductive Nutrition Development 41:71–77.

Andrabi, S. M. H. 2007. A review on reproductive biotechnologies for conservation of endangered mammalian species. Animal Reproduction Science 99:223–243.

Baruselli, P. S. 2018. Assisted reproductive technologies (ART) in water buffaloes. Animal Reproduction 15:971–983.

Baruselli, P. S. 2020. Embryo transfer in buffalo (*Bubalus bubalis*). Theriogenology 1:150:221–228.

Bengis, R. G. 1988. Wildlife diseases in South Africa: a review. Revue Scientifique et Technique 7:807–821.

Bielanski, A. 2016. Risk of transmission of Mycobacterium avium ssp. paratuberculosis by embryo transfer of in vivo and in vitro fertilized bovine embryos. Theriogenology 66:260–266.

Campanile, G. 2010. Ovarian function in the buffalo and implications for embryo development and assisted reproduction. Animal Reproduction Science 121:1–11.

Das, G. K. 1996. Efficacy of various collection methods for oocyte retrieval in buffalo. Theriogenology 46:1403–1411.

de Carvalho, J. 2019. Superstimulation prior to the ovum pick-up improves the in vitro embryo production in nulliparous, primiparous and multiparous buffalo (*Bubalus bubalis*) donors. Theriogenology 138:164–168.

Di Francesco, S. 2011. The effect of season on oocyte quality and developmental competence in Italian Mediterranean buffaloes (*Bubalus bubalis*). Animal Reproduction Science 123:48–53.

Di Francesco, S. 2012. Influence of season on corpus luteum structure and function and AI outcome in the Italian Mediterranean buffalo (Bubalus bubalis). Theriogenology 78:1839–1845.

Ferré, L. B. 2020. Recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods. Animal 14:991–1004.

Glanzmann, B. 2016. The complete genome sequence of the African buffalo (Syncerus caffer). BMC Genomics 17:1001.

Herold, F. C. 2004. Epididymal sperm from the African buffalo (*Syncerus caffer*) can be frozen successfully with AndroMed® and with Triladyl™ but the addition of bovine seminal plasma is detrimental. Theriogenology 61:715–724.

Hyttel, P. 1997. Oocyte growth, capacitation and final maturation in cattle, Theriogenology 47:23–32.

International Union for Conservation of Nature. 2019. *Syncerus caffer*. The IUCN Red List of Threatened Species 2019:e.T21251A50195031. Accessed on 14 Dec 2019.

Kątska, L. 1984. Number and quality of oocytes in relation to age of cattle. Animal Reproduction Science 7:451–460.

Kidson, A. 1998. The application of assisted reproductive technologies for the production of Cape Buffalo (*Syncerus caffer*) embryos. Thesis, University of Stellenbosch, Stellenbosch, South Africa.

Kock, M. D. 2012. Chemical and physical restraint of wild animals. A training and field manual for African species. International Wildlife Veterinary Services, Greyton, South Africa.

Kruip, T. A., J. Muskens, H. J. W. van Roermund, D. Bakker, and N. Stockhofe-Zurwieden. 2003. Lack of association of Mycobacterium avium subsp. paratuberculosis with oocytes and embryos from moderate shedders of the pathogen. Theriogenology 59:1651–1660.

Lambrechts, H. 1999. The effect of cryopreservation on the survivability, viability and motility of epididymal African buffalo (*Syncerus caffer*) spermatozoa. Theriogenology 52:1241–1249.

Leon-Quinto, T. 2009. Developing biological resource banks as a supporting tool for wildlife reproduction and conservation: the Iberian lynx bank as a model for other endangered species. Animal Reproduction Science 112: 347–361.

Li, F. 2007. Collection of oocytes through oransvaginal Ovum pick-up for in vitro embryo production in Nanyang yellow cattle. Reproductive in Domestic Animal 42:666–670.

Manik, R. S. 2002. Folliculogenesis in buffalo (*Bubalus bubalis*): a review. Reproduction, Fertility and Development 14: 315–325.

Miller, R. S. 2013. Diseases at the livestock-wildlife interface: status, challenges, and opportunities in the United States. Preventive Veterinary Medicine 110:119–132.

Nava-Trujillo, H. 2020. Reproductive performance of water buffalo cows: a review of affecting factors. Journal of Buffalo Science 9:133-151.

Penitente-Filho, J. M. 2015. Influence of corpus luteum and ovarian volume on the number and quality of bovine oocytes. Animal Science Journal 86:148–152.

Presicce, G. A. 2020. Efficacy of repeated ovum pick-up in Podolic cattle for preservation strategies: a pilot study. Italian Journal of Animal Science 19:31–40.

Purohit, G. 2003. Reproductive biotechnologies for improvement of buffalo: the current status. Asian-Australasian Journal of Animal Sciences 16:1071-1086.

Renwick, A. R. 2007. Bovine tuberculosis in southern African wildlife: a multi-species host-pathogen system. Epidemiology and Infection 135:529–540.

Schmidt, S. 2006. Histo-morphology of the uterus and early placenta of the African buffalo (*Syncerus caffer*) and comparative placentome morphology of the African buffalo and cattle (*Bos taurus*). Placenta 27:899–911.

Shaw, D. G. 1995. In vitro production of African buffalo (*Syncerus caffer*) embryos derived from follicular oocytes and epididymal sperm. Theriogenology 43:322.

Sianturi, R. G. 2002. Effect of collection technique on yield of bovine oocytes and the development potential of oocytes from different grades of oocytes. Indonesian Journal of Animal and Veterinary Sciences 7:188–193.

Singh S. 2001. Effect of the presence of corpus luteum on oocyte recovery and subsequent in vitro maturation and fertilization in buffaloes. Asian-Australasian Journal of Animal Sciences 14:1675–1677.

Takuma, T. 2010. Effects of season and reproductive phase on the quality, quantity and developmental competence of oocytes aspirated from Japanese black cows. Journal of Reproduction and Development 6:55-59.

Taylor, R. D. 1988. Age determination of the African buffalo, *Syncerus coffer* (Sparrman) in Zimbabwe. African Journal of Ecology 26:207–220.

Tschopp, R. 2021. Effect of bovine tuberculosis on selected productivity parameters and trading in dairy cattle kept under intensive husbandry in central Ethiopia. Frontiers in Veterinary Science 8:698768.

van der Heijden, E. M. D. L., A. O. Jenkins, D. V. Cooper, V. P. M. G. Rutten, and A. L. Michel. 2016. Field application of immunoassays for the detection of *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). Veterinary Immunology and Immunopathology 169:68–73.

Wang, Z. 2007. Effects of collection methods on recovery efficiency, maturation rate and subsequent embryonic developmental competence of oocytes in Holstein cow. Asian-Australasian Journal of Animal Sciences 20:496–500.

Wildt, D. E. 1997. U. S. Genome Resource Banks. BioScience 47:689–698.

Wildt, D. E. 2000. Genome resource banking for wildlife research, management, and conservation. ILAR Journal 41: 228–234.

Associate Editor: Laura Sánchez-Sáenz.

How to cite this article: Kosior, M. A., K. Y. Lyne, F. Salerno, B. Gasparrini, and G. Esposito. 2024. Effect of age, pregnancy, and tuberculosis status on oocyte parameters in African buffalo. Journal of Wildlife Management 88:e22541. https://doi.org/10.1002/jwmg.22541