TRADITION AND MODERNITY IN VETERINARY MEDICINE, 2024, vol. 9, No 2(17): 77–82

MORPHOLOGICAL CHARACTERIZATION OF OOCYTES IN AN ADULT FEMALE GRAY WOLF (*CANIS LUPUS*), A CASE REPORT

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(Submitted: 26 June 2024; Accepted: 10 October 2024; Published: 25 November 2024)

ABSTRACT

The study describes the morphometric characteristics of the ovaries, follicles, and oocytes of an adult female gray wolf. Macroscopic examination revealed ellipsoidal ovaries, which measured 9×18 mm and weighed 844 mg and 752 mg for the left and right ovaries, respectively. Two large follicles (4 mm) were prominent on the surface of the left ovary. The mean diameter of the collected primary oocytes (n=22) was 116.8±12.35 μm (including *zona pellucida*). Microscopic examination revealed dark, finely granulated, homogenous cytoplasm surrounded by a clearly visible *zona pellucida* and 2–5 layers of cumulus cells.

Limited scientific studies are available due to challenges in obtaining biological material. The present analysis offers preliminary insights into the morphological features of the gray wolf oocytes and is informative for future research aimed at species' conservation.

Key words: gray wolf, ovary, oocyte, morphology.

Introduction

The gray wolf (*Canis lupus*) is considered as an apex predator and keystone species because of its crucial role in ecosystem health. While the global decline in large apex consumers, existing for millions of years, is often seen ethically and aesthetically, recent research has uncovered far–reaching consequences, emphasizing the urgency of interdisciplinary studies to predict how their loss affects ecological processes, global ecosystem resilience, and socioeconomic effects on surrounding communities (Estes *et al.* 2011; Gregr *et al.* 2020). It is also important to highlight that preserving intact food chains, regardless of significant environmental disturbances, is of utmost importance (Wilmers et Getz 2005).

The challenges posed by the canine reproductive model, characterized by two ovulations per year, a small number of mature oocytes, and the need for an experimental kennel, have hindered the development of research efforts in reproductive biotechnology (Reynaud *et al.* 2020). To address this issue, the International Embryo Technology Society (IETS) established an advisory committee on companion animals, and non–domestic and endangered species to provide information and resources to facilitate the advancement of research in this area (CANDES, https://www.iets.org). Ovulation in domestic dogs can be monitored through various methods, including hormonal (LH) assays, vaginal cytology, and ultrasonography, although this is not feasible in wild animals. This study aims to contribute to this field by providing fundamental insights into overcoming these obstacles.

Based on reports by the European Federation of Hunting and Conservation (FACE), which focused on the degree of preservation of large carnivore species (brown bear, lynx, wolf, and wolverine) and the dynamics of individual populations, an increase in their range has been observed

over the past few years. Reports as of 2021 indicate that the gray wolf is now widely established and reproducing successfully, which, in turn, has led to a significant increase in the wolf population from 39% to 43%. This increase logically led to an expansion in species range. Similarly, in Europe, in the winter of 2018, there was an increase and re–establishment of the population of this species in areas where it had been absent for decades (https://www.face.eu/2022/12/the–wolf–at–the–bern–convention/). However, the positive trend in distribution range expansion alone is insufficient for a comprehensive evaluation of the overall status of the species. The preservation or recovery of gray wolves may be a crucial conservation necessity to sustain the adaptability of wildland ecosystems (Ripple et Beschta 2012). Assisted reproductive techniques are well– established for fertility preservation in intensive domestic breeding (Suzuki *et al.* 2022). Some of these techniques have also been effectively used as part of programs to maintain genetic diversity in wild populations. However, limited knowledge of the reproductive capacity of gray wolves renders them unsuitable, which complicates the accurate prediction of population numbers.

The aim of the present study was to morphologically and morphometrically examine the reproductive system of an adult female gray wolf, including the uterine horns, cervix, ovaries, and oocytes.

Materials and methods

Organs and tissues from the reproductive system of one female gray wolf (*Canis lupus*) with a weight of 42 kg and an estimated age of 3 years were obtained following an unplanned hunt on 24 February 2023 in the Bulgarian Rhodope Mountains.

The collected tissue was transported in a thermal box with a 0.9% NaCl solution to remove excess blood and maintain a moist ovarian cortex. Morphometric measurements were performed on uterine horns, cervix, ovaries, and oocytes.

Ovaries were excised from fat and other tissues using forceps and surgical scissors. After weighing both ovaries, the left ovary was processed by slicing its surface with an 11–mm surgical blade in a glass petri dish containing 25 mM HEPES–TCM199–Earle's salts medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (Gibco), 40 IU/ml heparin (Pisa), and 100 U–1 μg/ml penicillin–streptomycin (Sigma). After processing, cumulus–oocyte complexes (COCs) were collected using a stereomicroscope, washed twice with a clean medium to remove the blood and loose granulosa cells, observed, and measured.

The diameter of each oocyte (n=22) was measured using the Cronus camera program (Version 3.6, Research Instruments Ltd.), taking the average of two perpendicular lengths. Measurements included the diameters of the oocyte (with the *zona pellucida*) and the ooplasm. Before measurement using the software program, scale calibration was performed using an objective micrometer on a Nikon Eclipse Ti–U inverted microscope (Nikon Instruments Inc.).

After oocyte retrieval, ovaries were cut into pieces for slow freezing for future analysis.

The results are presented as mean ±SEM (where available). Mann Whitney U test on Statistica 6 (StatSoft) was used for statistical analysis. The significance level was set at $p < 0.05$.

All applied methods were performed in accordance with Bulgarian animal protection law (2022) and Regulation “Humane treatment of animals” (2023).

Results and Discussion

The macroscopic examination, followed by morphometric evaluation, demonstrated ellipsoidal ovaries, 9 × 18 mm in size. The weights of the left and right ovaries were 844 mg and 752 mg, respectively. The surface of the left ovary had two large follicles > 4 mm in diameter. In contrast, the right ovary had a smooth surface, with no prominent follicles (Figure 1). During the preovulatory stage, under luteinizing hormone stimulation, follicle growth accelerates, and protuberances may become visible prior to ovulation (Rocha *et al.* 2007).

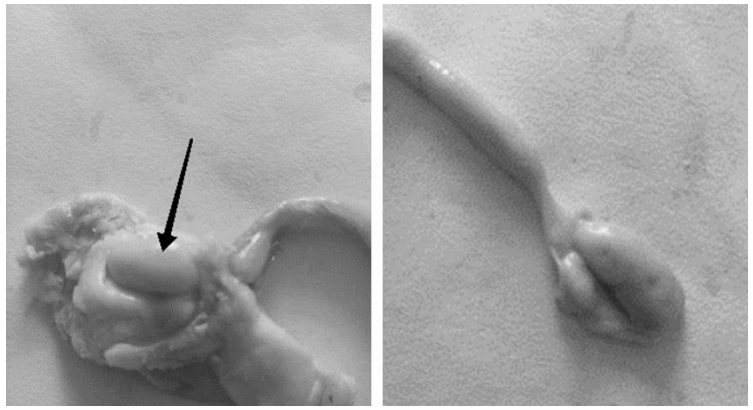

Figure 1: Gray wolf left (arrow at a prominent follicle) and right ovaries during the preovulatory stage.

Microscopic examination of the oocytes (n=22) revealed a dark, finely granulated, homogenous cytoplasm surrounded by a clearly visible zona pellucida and 2–5 layers of cumulus cells (Figure 2). Morphometric analysis revealed that the mean diameter of the oocytes (including zona pellucida) was 116.8 ± 12.35 μm and the diameter of the ooplasm was 78.58 ± 9.83 μm ($p = 0.022$). Despite the evident morphological similarity, the isolated gray wolf oocytes were smaller than some of the domestic dog oocytes measured in our previous study (Gradinarska *et al.* 2017). This suggests that there may be differences in oocyte development and size, with implications for the reproductive biology of the two subspecies and potential impact on fertility.

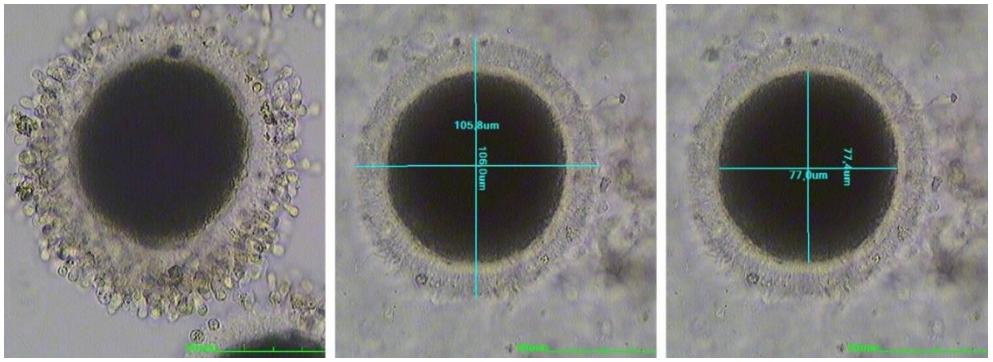

Figure 2: Inverted microscope images of COCs (left) and measurement of primary oocytes with and without *the zona pellucida* (right).

The genus Canis has a more complex egg maturation system than other species. In most mammals, gametes mature during or shortly after ovulation, whereas in dogs, it takes 48–72 hours to achieve nuclear maturation in the oviduct (Reynaud *et al.* 2005). This is an excessive time interval that may be related to the difficulties in inducing maturation *in vitro* (Songsasen et Wildt 2007). Ovulation in female dogs occurs when the oocytes are still immature, 1–2 days after the preovulatory LH peak. Follicles begin luteinization before ovulation; however, the fallopian tube in the genus Canis plays a crucial role in oocyte maturation. The oviduct environment supports immature oocytes until they reach the full development stage. After fertilization, the developing embryo is dependent on oocyte nutrient reserves and the oviduct environment to acquire the metabolites necessary for growth to the blastocyst stage (Luvoni *et al.* 2005). Oocytes are at the beginning of the first meiotic division and thus require 2–5 days for maturation (Rocha *et al.* 2007). This reduces the success of *in vitro* embryo production. Few studies have reported life birth from *in vitro*–fertilized embryos using *in vivo*–matured oocytes (Nagashima *et al.* 2015).

Morphologically, oocytes in the genus Canis are characterized by a high lipid content (phospholipids and triglycerides), which makes the ooplasm appear denser and darker (Hu *et al.* 2020). The number and morphological quality of collected oocytes depend on the age of the animal; therefore, donors aged 1–3 years are recommended (Lopes *et al.* 2007). In the present study, the isolated COCs from the ovary of the wolf demonstrated well–defined corona radiata, zona pellucida, vitelline membrane, and perivitelline space. The isolated complexes showed good cell–to–cell communication prior to ovulation. It has been suggested that the morphological characteristics of COCs are related to changes in oocyte metabolism and cell communication. The weakening of the connections between cumulus cells and gametes is chronologically related to oocyte maturation (Rodriguez et Farin 2004). Therefore, these connections play a major role in coordinating nuclear and cytoplasmic maturation. Additionally, COCs with an imperfect, incomplete layer of cumulus cells, and individual oocytes lacking cumulus cells were noted. Cumulus cells with a loose arrangement around the oocyte are thought to result in a decrease in oocyte quality, as their ability to advance meiosis and reach the MII stage relies on communication with cumulus cells (Haenisch–Woehl *et al.* 2003).

Oocyte diameter is one of the indicators of female gamete status. According to Otoi *et al.* (2000), diameter is directly related to meiotic competence, which determines gamete maturation rates during proestrus and estrus. It has been emphasized that the reproductive cycle is unique among other mammals in that females experience prolonged and variable periods of ovarian inactivity linked with waves of gamete development during folliculogenesis (Songsasen et Nagashima 2020; Barati *et al.* 2022).

In the future, it would be important to examine the effectiveness of cryopreserved oocyte maturation to determine if it is as effective in gray wolves as in dogs (Abe *et al.* 2008).

Conclusion

The morphological characterization of the gray wolf oocytes provides fundamental knowledge in terms of the small number of scientific studies, owing to the challenges of accessing biological materials. Further studies on oocytes from this species are needed to delve deeper into the estrus cycle and oocyte quality to enhance the understanding of female gametes in the genus Canis and to further contribute to the knowledge of the overall reproductive process. Moreover,

such information is likely to be useful for establishing an *in vitro* follicle culture system for genetic preservation in the event of potential threats to the species.

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