

Follicular fluid supports cumulus expansion in post-mortem recovered cumulus-oocyte complexes of *Hippopotamus amphibius*

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ABSTRACT

Assisted reproduction is essential for the conservation of wildlife species. While obtaining oocytes from living individuals can be challenging, the retrieval of post-mortem ovaries provides a valuable alternative for developing reproductive protocols that could enhance preservation efforts. In this study, we aimed, to evaluate the impact of follicular fluid (FF) supplementation during *in vitro* maturation (IVM) on nuclear maturation and cumulus expansion of common hippo (*Hippopotamus amphibius*) oocytes. Ovaries were recovered during the post-mortem examination of a 33-year-old female hippo, and 44 oocytes were collected and randomly allocated into three groups for IVM. A control group matured in standard media, a Small-FF group with control standard supplemented with 10 % follicular fluid from small follicles, and a Large-FF group with standard media supplemented with 10 % follicular fluid from large follicles. Images were taken at 0, 16, and 24 h of IVM to evaluate cumulus expansion by measuring the diameter of each cumulus-oocyte complex (COC). Following 24 h of IVM, polar body (PB) extrusion was assessed. Results showed a significant increase in cumulus expansion in the FF-supplemented groups compared to the Control (16 h: Control: 13 ± 4 %, Small-FF: 43 ± 13 %, Large-FF: 44 ± 6 %; 24 h: Control: 27 ± 7 %, Small-FF: 71 ± 15 %, Large-FF: 66 ± 9 %). The Small-FF group (46.1 %) and Control group (40 %) exhibited similar percentages of PB extrusion, while PB could be observed in 6.3 % of the Large-FF group. These findings suggest that while FF supplementation promotes cumulus expansion, the size of the follicle from which the fluid is derived may significantly influence oocyte ability to achieve PB extrusion

1. Introduction

While substantial knowledge exists regarding the reproduction of domestic animals, the scenario is markedly different for endangered wildlife species. For most of these species, documented information is scarce, and efficient protocols for assisted reproductive technologies (ARTs) have yet to be developed [1]. Understanding the anatomy, basic reproductive physiology and gonadal function of each species remains one of the most critical and limiting factors and a key point in advancing

this field [2]. Furthermore, the development of efficient protocols to cryopreserve the genetic resources of wildlife species plays an essential role in maintaining species integrity. ARTs can facilitate the distribution of valuable genetic material worldwide, ensure the preservation of existing genetic diversity, and expand the available knowledge on endangered species [3,4]. These benefits are crucial to supporting the conservation of endangered species, and are closely linked to bio-banking efforts [5,6].

Additionally, the considerable variations in cryobiological properties

Abbreviations: ANOVA, Analysis of Variance; ARTs, Assisted Reproductive Technologies; BSA, Bovine Serum Albumin; COCs, Cumulus-Oocyte Complexes; EGF, Epidermal Growth Factor; EVs, Extracellular Vesicles; FBS, Fetal Bovine Serum; FF, Follicular Fluid; GSH, Glutathione; IVF, *In Vitro* Fertilization; IVM, *In Vitro* Maturation; IUCN, International Union for Conservation of Nature; LPCs, Lysophosphatidylcholines; MII, Metaphase II; PB, Polar Body; PBS, Phosphate-Buffered Saline; ROS, Reactive Oxygen Species; SOD, Superoxide Dismutase; TCM-199, Tissue Culture Medium 199; TGF- β , Transforming Growth Factor Beta.

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between species and the maturation stages of the oocyte further complicate the development of standardized protocols, highlighting the need for species-specific approaches. As such, a specific cryopreservation protocol for each cell type and species should be developed [7]. Furthermore, it has been observed that mature oocytes yield better vitrification outcomes compared to immature oocytes in terms of oocyte quality and developmental competence [8]. When biological samples are difficult to obtain, recovering biological material from deceased animals, including gamete recovery, can be a valuable resource for the conservation of endangered species. Indeed, to date, several offspring have been produced using gametes recovered post-mortem [9].

One wildlife species that could potentially benefit from ARTs is the common hippopotamus (*Hippopotamus amphibius*), a large herbivorous mammal native to the southern regions of the Sahara Desert [10]. With wild populations declining, the International Union for Conservation of Nature (IUCN) lists hippos as vulnerable to extinction [11]. This indicates that their situation is not yet critical, but they are at risk of disappearing if conservation efforts are not effectively implemented. Female hippos reach puberty at around 9–10 years of age and are polyestrous and monotocous, with an estrous cycle duration of approximately 30–35 days based on hormone patterns [10,12]. While the reproductive cycle of this species is partially understood, knowledge of other aspects of their reproductive physiology is notably limited compared to other African herbivores like elephants and rhinos [10,12]. Increasing our understanding of reproductive aspects is essential in species conservation strategies. To our knowledge, no research has been conducted on the *in vitro* maturation of oocytes or their developmental competence in this species. Within this context, developing an optimized protocol for oocyte maturation is crucial for species conservation, as it would support genetic preservation and enhance breeding programs through assisted reproductive technologies.

Different strategies have been suggested to improve oocyte maturation rates across several species, for example maturation media can be supplemented with antioxidants, hyaluronic acid or follicular fluid (FF) [13–15]. As such, follicular fluid supplementation has been widely evaluated as an alternative approach to improve oocyte maturation in several species, although it has not been tested in hippos yet [16,17]. In the present study, we aimed to evaluate, for the first time, the impact of follicular fluid supplementation on polar body extrusion and cumulus expansion of cumulus-oocyte complexes (COCs) from the common hippopotamus (*Hippopotamus amphibius*).

2. Materials and methods

2.1. Chemicals and suppliers

Except where otherwise specified, all chemicals and reagents were supplied from Sigma-Aldrich (Merck, MA, USA).

2.2. Post-mortem recovery of the ovaries

The ovaries were removed during the routine necropsy of a 33-year-old multiparous *Hippopotamus amphibius* female housed at the Parc Zoològic de Barcelona. Following collection, the ovaries were preserved at 4–8°C under refrigerated conditions and sent to the laboratory within 24 h.

2.3. Macroscopic evaluation

Upon arrival at the laboratory, the ovaries were visually inspected to confirm their normal appearance and the presence of follicles in the ovarian cortex. Their size and weight were subsequently documented.

2.4. Microscopic evaluation

After oocyte recovery (see Section 2.5), the ovaries were fixed in

10 % neutral buffered formalin for subsequent histological examination. Once fixed, the ovaries were sliced, and four sections from different areas containing at least one visible antral follicle were selected and embedded in paraffin wax. Sections were cut with a microtome into 4–5 µm-thick slices and stained with hematoxylin and eosin following the manufacturer's protocol.

2.5. Follicular fluid aspiration and oocyte recovery

Follicular fluid from antral follicles measuring 1–4 mm (small), 5–10 mm (medium) and > 11 mm (large) in diameter was aspirated using an 18-gauge needle. After sedimentation, the supernatant from small and large follicles was kept for subsequent use (see Section 2.6). The pellets were resuspended in modified phosphate-buffered saline (PBS) supplemented with 0.036 mg/mL sodium pyruvate, 0.05 mg/mL gentamicin, and 0.5 mg/mL bovine serum albumin (BSA) for oocyte recovery. Subsequently, the ovarian cortex was meticulously sectioned into thin slices using a sharp scalpel in a dish filled with PBS. Special care was taken to ensure precision during the slicing process to preserve the integrity of the tissue. Following this step, the medium was carefully examined under a stereomicroscope to identify and recover the maximum possible number of COCs. This meticulous approach aimed to optimize the retrieval process and ensure a high yield of viable COCs for further analysis or experimental procedures.

2.6. *In vitro* maturation

COCs with homogenous cytoplasm were washed three times in PBS and subsequently transferred in groups of 13–16 COCs to 30 µL drops of maturation media composed of Tissue culture medium-199 (TCM-199) supplemented with 10 % (v/v) fetal bovine serum (FBS), 10 ng/mL epidermal growth factor, and 50 mg/mL gentamicin. The recovered oocytes were randomly divided into three experimental groups: (1) Control: maturation media without supplementation (n = 15), (2) Small-FF: maturation media supplemented with 10 % follicular fluid from small follicles (n = 13) and (3) Large-FF: maturation media supplemented with 10 % follicular fluid from large follicles (n = 16). Oocytes were subjected to *in vitro* maturation (IVM) for 24 h at 37°C in a 5 % CO₂ humidified atmosphere. At the end of the IVM and after further evaluation (see Sections 2.7 and 2.8), all oocytes were vitrified following a short equilibration protocol [18] and stored in liquid nitrogen as part of the Barcelona Zoo Biobank.

2.7. Cumulus cell expansion

Images of COCs from each experimental group were captured using an Olympus EP50 camera (Olympus soft imaging solutions, Münster, Germany) mounted on a stereomicroscope at 2.5x magnification at three different timepoints: (1) prior to IVM, (2) after 16 h of IVM and (3) after 24 h of IVM. The images were analyzed using ImageJ software (version 2.14.0/1.54 f) to measure the area of the COCs. Cumulus cell expansion was quantified as the percentage increase in COC area between consecutive timepoints.

2.8. Polar body extrusion

To assess polar body extrusion, COCs were gently and partially denuded by pipetting in PBS after 24 h of IVM. Each oocyte was examined for the presence or absence of a polar body, indicating whether nuclear maturation progression had occurred.

2.9. Statistical analysis

Statistical analysis and visualization of graphs were performed using GraphPad Prism 9 (GraphPad Software, CA, USA) and R software V 4.1.1 (R Core Team, Vienna, Austria). Normality and homogeneity of

variances were assessed with Saphiro-Wilk and Levene's tests, respectively. One-way ANOVA with Bonferroni post-hoc tests was conducted for group comparisons in cumulus expansion analysis. Results are presented as mean $\pm$ standard error of the mean (SEM) with significance set at $p \leq 0.05$.

3. Results

3.1. Macroscopic examination and follicular fluid collection

As shown in Fig. 1, the ovaries of the hippopotamus female exhibited a normal appearance with the presence of numerous antral follicles protruding from the ovarian surface. The ovaries measured 6×5 cm and 6×4 cm, with respective weights of 41.3 g and 32.8 g. A total of 8.3 mL of follicular fluid was collected, with 3.9 mL obtained from small follicles, 1.8 mL from medium follicles and 2.6 mL from large follicles.

3.2. Microscopic examination

All the examined sections displayed normal histological architecture, with no significant pathological findings. Multiple follicles at various stages of development were identified. The most frequently observed were primordial and primary follicles (Fig. 2A), followed by large antral follicles (Fig. 2B), many of which exhibited varying degrees of degeneration, including detachment of cells into the antrum (atretic follicles).

3.3. Cumulus cell expansion

According to Fig. 3, after 16 h of IVM, both Small-FF and Large-FF groups showed significantly increased cumulus expansion (43 ± 13 % and 44 ± 6 %, respectively) compared to the Control group (13 ± 4 %). Similar results were obtained after 24 h of IVM, with the Small-FF and Large-FF groups achieving cumulus expansion levels of 71 ± 15 % and 66 ± 9 %, respectively, both significantly higher than the Control group (27 ± 7 %).

3.4. Polar nuclear extrusion

The percentage of oocytes presenting a visible polar body after 24 h of IVM varied among the treatment groups. The Small-FF group exhibited the highest PB extrusion (46.1 %, $n = 6/13$), followed by the Control group (40 %, $n = 6/15$). In contrast, the Large-FF group showed a markedly lower PB extrusion rate (6.3 %, $n = 1/16$) as illustrated in

Fig. 4.

4. Discussion

The ability to mature oocytes is crucial for obtaining competent oocytes that can be cryopreserved in biobanks, serving as an essential tool for developing ARTs in endangered species. Successful oocyte maturation marks the first step toward *in vitro* embryo production followed by embryo transfer and somatic cell nuclear transfer programs [19].

The present study represents the first documented attempt to perform IVM of oocytes from *Hippopotamus amphibius*, demonstrating that a standard maturation media, TCM-199 supplemented with EGF and FBS (see Section 2.6), successfully achieved rates of PB extrusion of 40 % after 24 h of culture. While these rates are lower than the approximately 80 % of nuclear maturation typically reported in well-characterized domestic species such as bovine and porcine [18,20], they are consistent with maturation rates described in other species. For instance, in equine, domestic cat and southern white rhinoceros maturation rates range from 37 to 45 % [21–23]. Furthermore, the observed rates are higher than those reported in whales, the closest extant relatives to hippopotamuses, which exhibit maturation rates ranging from 26.7 % to 31.8 % [24]. These findings represent a promising starting point for future reproductive research and conservation efforts in common hippo.

Moreover, follicular fluid supplementation during IVM positively influenced cumulus cell expansion. Since follicular fluid is the natural environment where oocyte maturation occurs, its supplementation in *in vitro* maturation systems has been widely studied in multiple species. Consistent with our findings, enhanced maturation rates have been reported in canine, equine and porcine oocytes supplemented with follicular fluid during IVM [25–27], and improved developmental competence was observed in bovine and porcine species [26,28]. However, no effect was found when supplementing IVM media with follicular fluid in alpacas and jennies regarding maturation or development outcomes [16,29]. In terms of cumulus cell expansion, a negative effect was observed in donkeys [29], whereas bovine studies demonstrated increased cumulus cell expansion [28,30]. Moreover, the improvement in cumulus cell expansion was correlated with higher fertilization rates when COCs were subjected to *in vitro* fertilization (IVF) [30]. These inconsistent results across studies may be attributed to species-specific differences, variations in IVM protocols and supplementation percentages, follicular fluid processing methods or disparities



Fig. 1. Macroscopic image of the *Hippopotamus amphibius* ovaries in their anatomical position showing normal appearance with the presence of follicles (white arrowheads) protruding over the ovarian surface. Scale in cm.

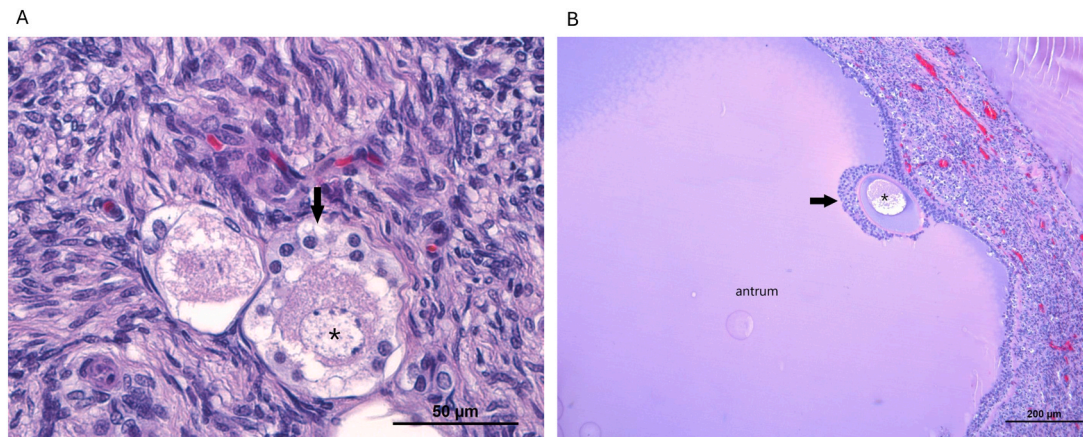


Fig. 2. Histological sections of the *Hippopotamus amphibius* ovaries. (A) Primary follicle with an oocyte (asterisk denotes the germinal vesicle) surrounded by a single layer of cuboidal granulosa cells (black arrow) (magnification 40 ×); (B) Large antral follicle with the oocyte (asterisk denotes the germinal vesicle) surrounded by the cumulus oophorus (black arrow) and a large fluid-filled antral cavity (magnification 10 ×).

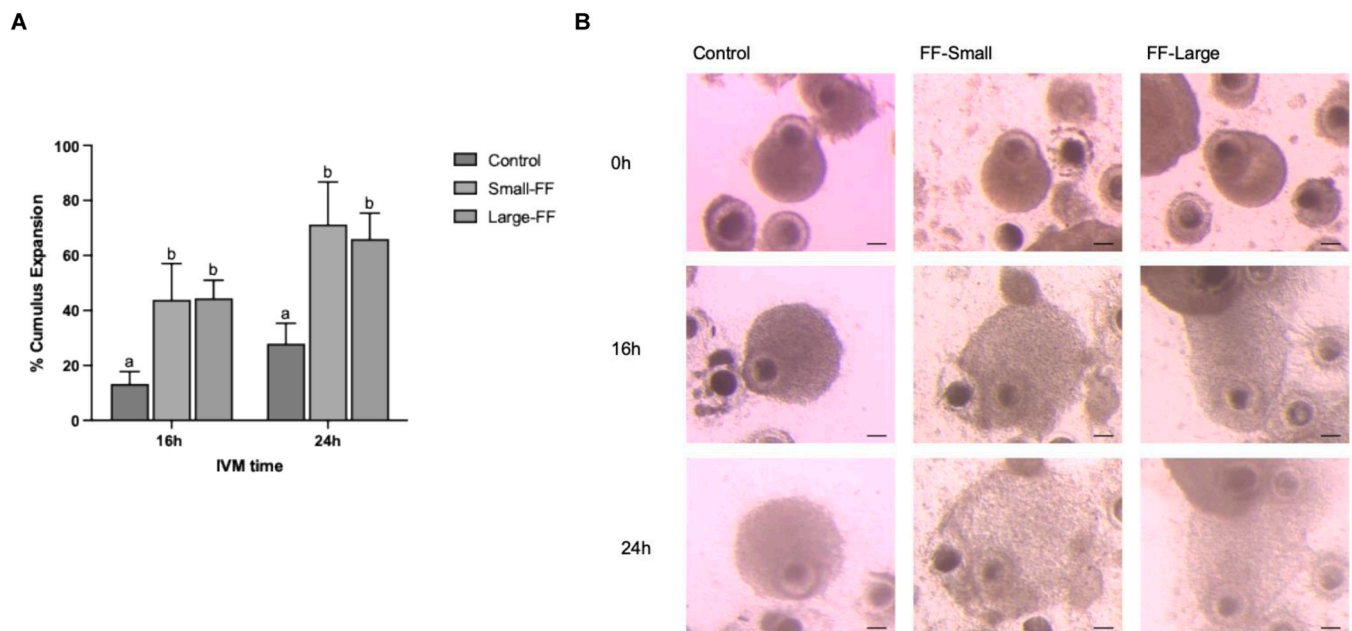


Fig. 3. Effects of follicular fluid supplementation on cumulus expansion at 0 h, 16 h and 24 h of IVM in hippo oocytes. (A) Percentage of cumulus expansion. Cumulus cell expansion was quantified as the percentage increase in COC area between consecutive timepoints. Data are presented as mean $\pm$ SEM. Bars with different letters (a, b) within the same time point indicate statistically significant differences ($p < 0.05$). (B) Images of hippo cumulus-oocyte complexes at each time point under the three experimental conditions. Control: maturation media without FF supplementation; Small-FF: maturation media supplemented with 10 % follicular fluid from small follicles; Large-FF: maturation media supplemented with 10 % follicular fluid from large follicles. Scale bar, 150 μ m.

in follicle sizes. Follicular fluid supplementation can lead to cumulus expansion due to the presence of key bioactive components such as EGF, which enhances proteoglycan synthesis [31], and Transforming growth factor beta (TGF- β) [32] which stimulates hyaluronic acid production [33]. Additionally, extracellular vesicles (EVs) present in follicular fluid have been shown to independently induce cumulus expansion and viability by influencing genes related to the MAPK pathway [34].

In the present study, follicular fluid derived from small follicles showed the greatest benefits, enhancing cumulus expansion without compromising nuclear maturation rates, while follicular fluid derived from large follicles exhibited a detrimental effect on maturation rates. A comparative metabolomic study between cow, sow and mare revealed species-specific and size-dependent variations in energy substrates of the follicular fluid. As follicle size increased, glucose levels were elevated in the cow and mare, while lactate levels decreased in the cow. The highest

differences between follicle sizes were found in monovulatory mammals [35]. Consistently, other studies reported that glucose and cholesterol concentrations increased from small to large follicles, while potassium, lactate and triglyceride levels decreased in bovine [36] and ovine [37]. Additionally, higher proportions of lysophosphatidylcholines (LPCs), lipids associated with inflammation and apoptosis, cholesterol esters, reactive oxygen species (ROS), glutathione (GSH) levels and superoxide dismutase (SOD) activity between small and large follicles have been described [38,39]. Furthermore, proteomic analyses in the porcine species revealed that small follicles are enriched in proteins that favor oocyte maturation, while large follicles contain proteins associated with the preparation for ovulation, and pathway analysis identified estrogen and PI3K-Akt signaling as predominant in small follicles, while complement and coagulation cascade pathways were significantly represented in large follicles [40].

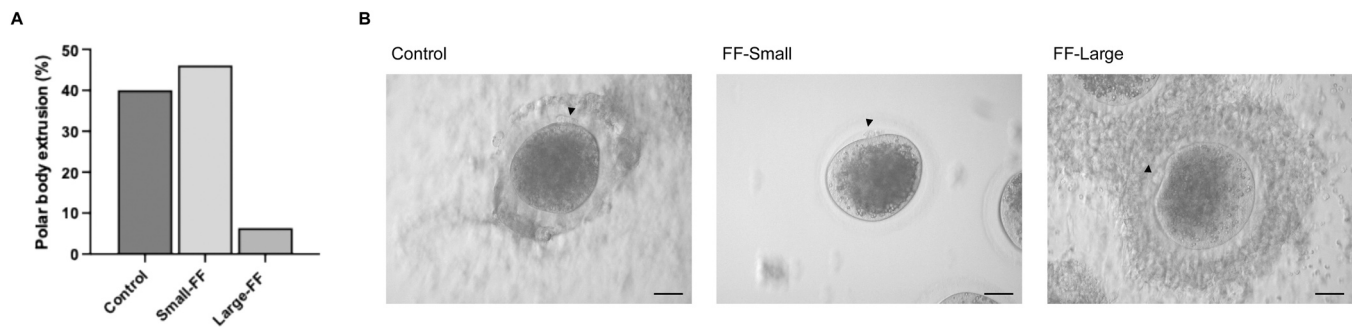


Fig. 4. Effects of follicular fluid supplementation on PB extrusion of hippo oocytes during IVM. (a) percentage of oocytes showing pb extrusion. (b) Representative images of oocytes showing PB extrusion. Black arrowheads indicate the first polar body. Control: maturation media without FF supplementation; Small-FF: maturation media supplemented with 10 % follicular fluid from small follicles; Large-FF: maturation media supplemented with 10 % follicular fluid from large follicles. Scale bar, 50 μ m.

These size-dependent variations are likely to influence follicular fluid ability to support oocyte maturation and may explain the differences in maturation outcomes observed in the present study. For instance, in equine, elevated glucose levels have been linked to reduced mitochondrial function, affecting ATP production and increasing non-mitochondrial respiration [41]. Moreover, excessive glucose can disrupt metabolic key pathways such as glycolysis and the pentose phosphate pathway, ultimately impairing oocyte competence by compromising cytoplasmic maturation and preventing the completion of nuclear maturation [42]. Similarly, excessive ROS levels can negatively impact oocyte quality and developmental competence. The production of ROS is an inevitable process in *in vitro* culture systems [43]. The ROS generated during *in vitro* maturation, combined with the potential additional ROS from follicular fluid derived from large follicles, may contribute to an imbalance in the cellular redox state, leading to oxidative stress. Studies in rats have shown that oxidative stress can inhibit first polar body extrusion [44].

Although the composition of *Hippopotamus amphibius* follicular fluid and the specific differences between small and large follicles remain unknown, similar results to those observed in other species can be expected. To fully elucidate the mechanisms underlying the observed differences in maturation outcomes, future studies should focus on characterizing the molecular composition of follicular fluid from different follicle sizes. Assessing the presence of growth factors, extracellular vesicles, key metabolic substrates and lipids would provide insights into the observed differences in maturation outcomes and could help clarify the metabolic requirements for oocyte maturation in this species.

This study was conducted using ovaries collected opportunistically, which inherently limited the sample size. While this constraint may affect our results, reporting these data remains crucial for lesser-studies species, as any new information contributes to the refinement of assisted reproductive techniques and conservation strategies. Future studies with larger sample sizes will be necessary to validate these results and assess potential variability, ensuring a more comprehensive understanding of oocyte *in vitro* maturation in this species.

5. Conclusions

The present study demonstrates that supplementing IVM media with follicular fluid from small follicles significantly enhances cumulus-oocyte complex expansion during *in vitro* maturation of *Hippopotamus amphibius* oocytes, without compromising nuclear maturation rates. Conversely, the use of follicular fluid from large follicles markedly reduces nuclear maturation rates. These findings represent a pivotal step toward optimizing *in vitro* maturation protocols for hippo oocytes, providing valuable insights for the development of ARTs aimed at preserving endangered species. By advancing our understanding of species-

specific reproductive physiology, this study lays the groundwork for innovative conservation strategies and reinforces the importance of tailored approaches in wildlife biobanking and assisted reproduction.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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CRediT authorship contribution statement

Serres-Corral Paula: Writing – review & editing, Investigation, Formal analysis. **Gago Sonia:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Diaz-Muñoz Judith:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **López-Béjar Manel:** Writing – review & editing, Validation, Supervision, Funding acquisition. **Mogas Teresa:** Writing – review & editing, Validation, Supervision, Funding acquisition. **Almagro Vanessa:** Writing – review & editing, Resources. **Oliveras-Seguí Bruna:** Writing – review & editing, Formal analysis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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