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Original Research Article

Embryo developmental and morphokinetic patterns in the domestic cat (*Felis catus*): associations with maternal age and SRT1720 supplementation during oocyte *in vitro* maturation

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ABSTRACT

This study describes embryo developmental progression and morphokinetic patterns in embryos derived from oocytes of young and aged domestic cats, a model for endangered felids, using a time-lapse monitoring system. Because maternal aging is associated with reduced oocyte competence, possibly linked to mitochondrial dysfunction that may impair fertilization and embryo development, the potential influence of the SIRT1 activator SRT1720 during *in vitro* maturation on embryo developmental progression was also evaluated under these conditions. Oocytes from young (1.13 ± 0.79 years) and old (7.0 ± 1.10) cats were matured *in vitro* with SRT1720 (1-μM, 0.1-μM, and 0.05-μM) and without, followed by *in vitro* fertilization. 8-Day embryo *in vitro* culture was noninvasively monitored using the MIRI® time-lapse system.

Embryos from aged cats exhibited accelerated progression up to the morula stage; in contrast, embryos from young cats showed faster progression at later preimplantation stages and reached the hatched blastocyst stage. In young queens, embryos exposed to 0.1-μM showed numerically higher first cleavage and morula formation rates than controls, while 1-μM was associated with altered first cleavage timing and short cell cycles. In older queens, 0.1-μM maintained cleavage timing patterns comparable to untreated embryos but fail to produce hatched blastocysts, whereas 1-μM yielded a single hatched blastocyst despite signs of altered cell cycle dynamics.

Overall, SRT1720 supplementation did not significantly influence embryo development under the tested conditions. Morphokinetic monitoring provided descriptive observations suggesting distinct age-related developmental profiles. These findings provide baseline information on feline developmental kinetics and may support further investigation of metabolic modulators in feline assisted reproduction.

1. Introduction

Female mammalian fertility declines with advancing age, characterized by diminished ovarian function that reduces both oocyte quantity and quality, ultimately leading to decreased success rates in natural conception and assisted reproductive technologies [1–5]. With advancing maternal age, oocytes exhibit progressive deterioration characterized by defects in chromatid separation, abnormal chromosome decondensation, and spindle apparatus dysfunction. These age-related cellular abnormalities cause chromosomal misalignment during meiosis, resulting in aneuploidy that leads to embryonic lethality, spontaneous abortion, or congenital disorders [5–11].

Among the cellular alterations associated with reproductive aging, mitochondrial dysfunction has been widely implicated as a contributing factor to reduced oocyte competence [12].

Aging increases the mitochondrial susceptibility to oxidative stress and induces damage in other oocyte organelles and consequently compromising its fertility potential [13,14]. In aged mammals, oocytes experience decreased mtDNA levels and declining mitochondrial function, resulting in reduced ATP availability, diminished fertilization potential, impaired embryo development quality and suboptimal implantation and placentation rates [15–25].

Despite the reproductive limitations associated with aging, there is an urgent need to utilize older individuals for breeding in conservation

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programs, particularly for members of the *Felidae* family. All felids, except for the domestic cat, are currently listed on the "Red List of Threatened Species" (https://www.iucnredlist.org/). Accordingly, given the limited availability and high biological value of reproductive material from endangered species, strategies to preserve oocyte competence in aged females, together with approaches that enable non-invasive assessment of embryonic developmental progression, are particularly relevant for improving assisted reproductive outcomes in felid species.

In this context, time-lapse monitoring has emerged as a valuable non-invasive approach for continuous assessment of embryos during *in vitro* culture. By enabling uninterrupted observation under stable culture conditions, this technology allows detailed annotation of cleavage timing and developmental events without repeated embryo handling. These morphokinetic parameters provide a more comprehensive characterization of preimplantation development than conventional endpoint morphology alone and may reveal subtle differences associated with maternal age or experimental interventions. The clinical relevance of time-lapse imaging has been widely explored in human assisted reproductive technologies [26].

Ongoing efforts employing time-lapse imaging are expanding knowledge of morphokinetic patterns in the domestic cat (*Felis catus*) [27–30]. Beyond their relevance to domestic cat reproductive biology, these studies are particularly valuable because the species serves as an established model for developing assisted reproductive approaches applicable to wild counterparts [31].

Pharmacological activation of sirtuin pathways has been proposed as a potential strategy to mitigate age-related decline in oocyte competence. SRT1720 is a synthetic sirtuin-activating compound (STAC) of SIRT1, reported to be approximately 1000-fold more potent than resveratrol [32]. When used as a supplement during *in vitro* maturation, SRT1720 has been shown to enhance oocyte maturation, fertilization rates, and blastocyst formation in an aged mouse model [33]. Its effects are associated with regulation of cellular senescence, apoptosis, metabolism, oxidative stress, and inflammation [34,35], as well as modulation of PGC-1α, a key regulator of mitochondrial biogenesis that supports mitochondrial oxidative capacity by enhancing OXPHOS gene expression and stimulating mtDNA replication [36–38]).

Despite the growing interest in metabolic modulators in reproductive biology, little is known about how such compounds influence the developmental dynamics of embryos derived from aged oocytes, particularly in carnivore species. In this context, time-lapse monitoring provides a non-invasive method for continuous assessment of embryo development, enabling detailed analysis of cleavage dynamics and developmental timing. These morphokinetic parameters provide descriptive information on embryo developmental progression and may contribute to understanding age-related variation during preimplantation development without requiring invasive manipulation.

The present study aimed to characterize embryonic developmental competence and morphokinetic patterns in embryos derived from oocytes of young and aged domestic cats using a time-lapse monitoring system, and to evaluate whether supplementation with different concentrations of SRT1720 during *in vitro* maturation influenced embryonic developmental progression under the conditions tested.

2. Materials and methods

Ethical approval was not required for this study, as it was conducted using surgical waste tissues, and no animals were handled specifically for research purposes. All chemicals and reagents were purchased from Sigma Aldrich, Poland, unless otherwise stated. All procedures involving ovaries, oocytes, and embryo manipulation were performed under strict light deprivation conditions. A controlled surface temperature of 37.5°C was maintained, and only the microscope and laminar flow chamber lighting were used to preserve the integrity of the results.

2.1. Ovaries and oocyte collection

Ovaries were obtained from pure and mixed-breed, sexually mature, privately owned and colony queens, during routine ovariectomy or ovariohysterectomy and divided into 2 groups: ovaries from young queens (under 3 years of age) (n = 37) and from old queens (above 6 years of age) (n = 6) [39]. After surgical removal, ovaries were stored in transport media (PBS with 1% of Antibiotic Antimycotic Solution—10, 000 units of penicillin, 10 mg streptomycin and 25 μg amphotericin B *per* mL) at 4°C for no longer than 24h until Cumulus-Oocyte Complexes (COCs) recovery. Ovaries were placed into a Petri dish containing ovum pick-up (OPU) medium and carefully sliced with a #10 scalpel blade for COCs collection. COCs were classified under a dissecting microscope, according to Wood & Wildt [40]. Grade I and II oocytes were washed three times in Washing Media (WM).

2.2. In vitro maturation

The selected COCs from each culture were randomly subdivided into four groups according to the treatment: Control [Equine Oocyte Maturation Medium (EqIVM™) commercial medium only], SRT1720 1-μM, SRT1720 0.1-μM, SRT1720 0.05-μM in EqIVM ™. The wells were covered with mineral oil and matured for 24 h at 38.5°C in 5% CO_2 in air with maximum humidity.

2.3. In vitro fertilization

Semen used for *in vitro* fertilization was obtained from sexually mature tom cats (n = 8), undergoing routine orchiectomy procedures at veterinary clinics. Donor animals included both purebred and mixed-breed cats originating from privately owned households and colony populations. Semen retrieval was performed using urethral catheterization, following intramuscular administration of medetomidine [41], with transscrotal stimulation of the testes and epididymides [42]. In some cases, spermatozoa were also retrieved from the cauda epididymis following orchiectomy and subsequently cryopreserved according to the protocol described by Niżański [43]. Frozen semen straws were thawed in a water bath at 37°C for 30 s, then washed in Semen Preparation Medium (BO-SemenPrep), followed by centrifugation centrifuged at 35, 000 rpm for 5 min. Semen quality was evaluated using the Ceros II Sperm Analysis System® and samples meeting the minimum criteria of 1×10^6 motile spermatozoa/mL, 30% progressive motility, and 50% subjective motility were used for *in vitro* fertilization. After 24 h of oocyte maturation, COC were washed in EqIVM™ and placed in 300 μL EqIVM™. Subsequently, 100 μL of sperm suspension containing 1×10^6 motile spermatozoa/mL was co-incubated with COCs of for 18-24 h at 38.5°C in 5% CO_2 in air with maximum humidity.

2.4. In vitro culture and embryo development in the time-lapse system

After 18-24 h, presumptive zygotes from each culture were washed three times and transferred to Culture Coin® Embryo culture Dish for the MIRI® TL system. Each Culture Coin®, containing 14 wells for embryo culture, was prepared and calibrated in advance in the MIRI® TL with 25 μL of EqIVC-1™ medium *per* well and covered with 4 mL of mineral oil. The dishes were individually labeled and positioned within the TL system, which was programmed to capture images of embryos at 20-min intervals throughout their *in vitro* development. The time-lapse incubator was maintained at 38.5°C under 5% CO_2 in air with maximum humidity, and images were captured every 20 min across multiple focal planes.

On day 5, embryos showing signs of development were selected, washed and transferred into a new Culture Coin®, with EqIVC-2 ™ media, until the end of the *in vitro* culture period (*i.e.*, 199 h). Non-developed or degenerated zygotes were discarded at the time of medium change but were still considered for statistical analysis.

2.4.1. Embryo evaluation

Embryo development was assessed based on morphological and morphokinetic characteristics recorded by MIRI® TL. Embryo development was evaluated by the same experienced observer. The embryonic developmental stages were categorized as follows: two (2B), four (4B) and eight 8B) blastomeres; early- (EM), mid- (MM) and late-morula (LM); compacted (CB), expanded (EXPB), escaping (ESCB) and hatched blastocyst (HB) [29,44–50]. In cats, the formation or disappearance of pronuclei in oocytes cannot be visualized due to their dark cytoplasm [29]. Hence, the first cleavage was the parameter used to assess successful fertilization.

2.5. Experimental design

This study was designed to characterize embryonic developmental progression and morphokinetic patterns in embryos derived from oocytes of young and aged domestic cats using a time-lapse monitoring system, and to assess whether maternal age and SRT1720 supplementation during *in vitro* maturation influenced developmental outcomes over 8 days of culture.

Oocytes were collected opportunistically from routine neutering procedures and processed in independent IVF sessions conducted throughout the study. In each session, recovered COCs were randomly allocated to treatment groups according to SRT1720 concentration (0, 0.05, 0.1, and 1 μM) and maternal age class [young (Y) vs. old queens (O)]. Treatment groups were identified by combining the age class letter with the SRT1720 concentration, while untreated groups were labeled using the letter C (control). The untreated control group (YC) served as the study reference. These oocytes were under ideal conditions—despite acknowledging intra-species variability, seasonal influences and other factors that may influence oocyte and embryo performance [51,52] as well as the lower success rates of *in vitro* conditions, compared to *in vivo* [45,53–55]. For older queens, the groups were labeled as O0.05, O0.1, O1, and OC, following the same labelling rationale.

Because oocytes were obtained from clinical waste material, the number of oocytes available *per* treatment group varied among IVF sessions and consequently affected the number of presumptive zygotes available for culture in each group. Presumptive zygotes obtained during independent IVF sessions were cultured according to maternal age group and SRT1720 concentration. Day 0 was defined as the day of IVF. Embryos were cultured in the time-lapse system and monitored for 199 h (8 days) to evaluate morphokinetic parameters. All recordings were assessed by the same observer to ensure consistency in annotation.

Presumptive zygotes that did not undergo the first cleavage by 48 h post-insemination (hpi) were not considered cleaved in subsequent analyses. Because some embryos underwent the first, second and/or third cleavages before the initiation of TL recording, precise division timings were not available. Consequently, only presumptive timings (B2<20 hpi; B4<15 hpi; B8<10 hpi) are reported and discussed [45] and no average hours were calculated for these stages (identified as “ND-non defined”).

2.6. Statistical analysis

Statistical analyses were performed using R 4.5.2 (R Core Team (2025). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org. Data distribution normality was assessed using the Shapiro – Wilk test. Equality of variances in the analyzed groups was verified using Bartlett's test. The significance of the effects of age group and concentration was assessed using a two-way ANOVA, given by the model:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk},$$

where y_{ijk} is the value of the k-th observation in the i-th age group ($i = 1, 2$) and j-th concentration group ($j = 1,2,3,4$), μ is the overall mean, α_i is the age group effect, β_j is the concentration group effect, $(\alpha\beta)_{ij}$ is the interaction of the age and concentration groups, ε_{ijk} is the random error. In cases where the ANOVA assumptions were not met, the Kruskal–Wallis test was applied, with the interaction of the grouping factors treated as the effect (due to the independence of the factors). The significance of the effect of concentration on hatched blastocyst out of IVC in the young group, due to the lack of normality in the distribution of the variable, was assessed using the Kruskal–Wallis test. Groups in which the variables exhibited zero variability were not included in the respective statistical analyses.

A significance level of $P < 0.05$ was considered. Data are presented as mean ± standard error of the mean (SEM) or mean ± standard deviation (SD), as indicated in the corresponding sections.

The experimental design involved multiple culture replicates obtained from oocytes collected from different donors over the course of the study. Each IVF session constituted an independent culture event, and embryos obtained within each session were analyzed according to treatment group and maternal age class. Each replicate consisted of a group of presumptive zygotes cultured under identical experimental conditions and monitored using the time-lapse system. Due to the limited availability of ovaries from aged queens and the exploratory nature of the study, the statistical analyses focused primarily on identifying trends in developmental outcomes and morphokinetic parameters among treatment groups.

3. Results

The average ages were 1.13 ± 0.79 years for young cats and 7.00 ± 1.10 years for older queens. A total of 80 presumptive zygotes from young queens and 60 from older queens, all of high quality, were monitored using the TL system. Embryo development was captured every 20 min across seven focal planes, generating a dataset of 750,000 sequenced images. Key developmental milestones were identified and documented, as illustrated in Fig. 1.

Analysis of this comprehensive dataset revealed descriptive temporal patterns in embryo development. Sixty-six percent of our recorded embryos had already undergone the first, second, or third cleavage before the onset of TL recording. In both the experimental and control groups of young and old queens, the majority of first cleavage events occurred within less than 20 hpi (Fig. 4).

Despite similar early cleavage timing across both age groups, time-lapse monitoring documented distinct patterns of developmental progression during subsequent stages. Although no statistically significant differences were detected, descriptively, a greater proportion of embryos from older queens progressed through cleavage to the morula stage, and morula formation was also recorded earlier than in embryos from young queens. From the CB onwards, a higher proportion of embryos from young queens continued progression through subsequent developmental stages, and these stages were recorded earlier than in embryos from older queens. No embryos from older cats developed into HB, whereas 18% of fertilized oocytes from young cats reached this stage (Table 1, Fig. 2).

Furthermore, 44% of embryos that reached the early-morula stage in the older queens' control group underwent rapid divisions, reaching the morula stage before 72 hpi, in contrast to 25% of embryos from the young queens’ control group (Table 2).

These observations were used to describe patterns of cleavage timing in the study population. In the young cat's group, 58% of embryos cleaved before 20 hpi. Two-thirds of compacted blastocysts and half of the hatched blastocysts arose from embryos cleaving within that time frame. Based on these findings, 20 hpi was used as a descriptive reference point for the first cleavage time in cat embryos. In the old-cat group, 44% of embryos cleaved before 20 hpi, and 75% of compacted blastocysts arose from them (Fig. 4).

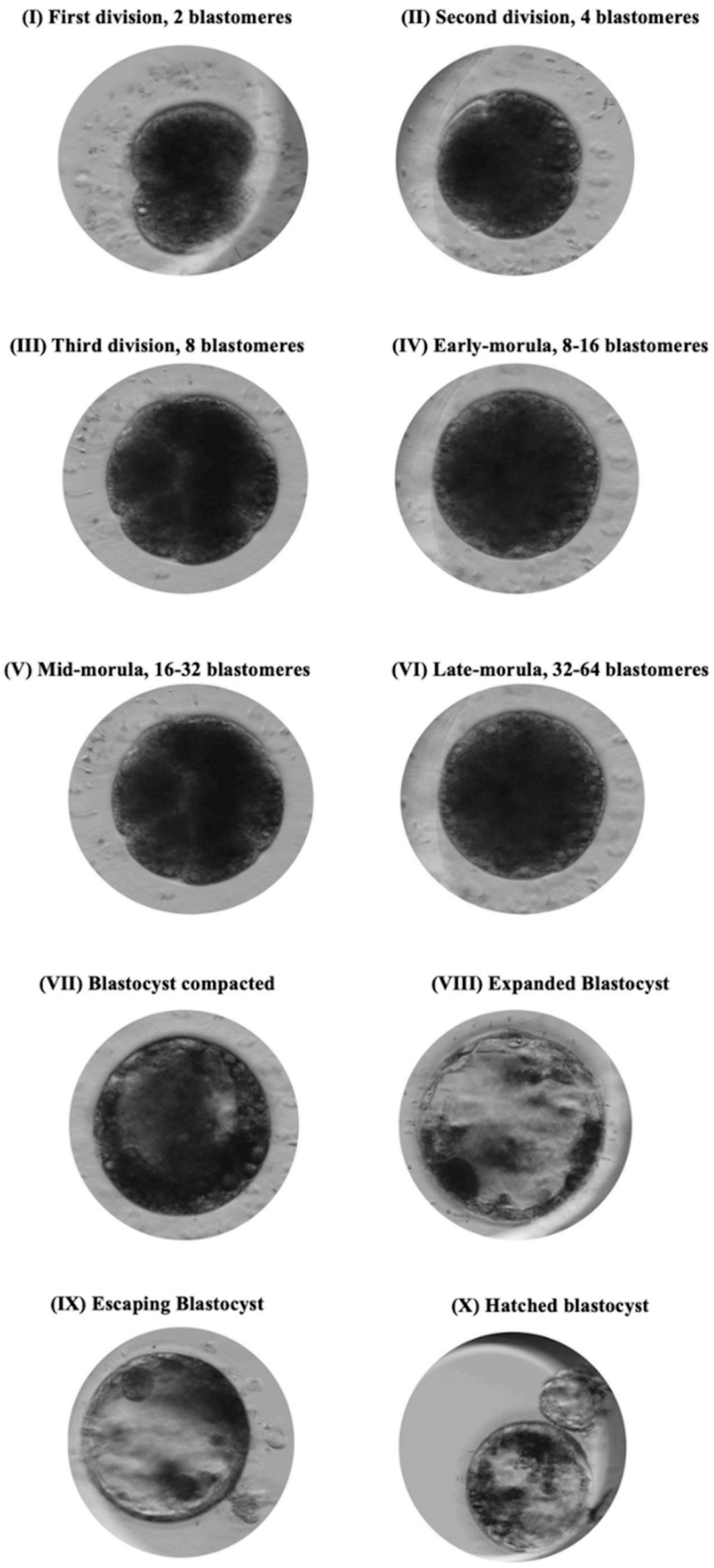


Fig. 1. Domestic cat embryo milestones evaluated in the time-lapse system.

Table 1
Influence of SRT1720 concentrations on embryo development milestones (cleavage, morula, compacted and hatched blastocyst stage).

Embryo stages	SRT1720 Treatment							
	Young queens				Old queens			
	C	1-μM	0.1-μM	0.05-μM	C	1-μM	0.1-μM	0.05-μM
Oocytes subjected to IVF (n)	**43**	**33**	**30**	**34**	**18**	**13**	**24**	**15**
Presumptive zygotes for IVC (n)	**22**	**17**	**19**	**22**	**14**	**13**	**21**	**12**
Cleaved (n)	**12**	**12**	**15**	**11**	**9**	**8**	**11**	**7**
% out of IVC	54.00 ± 15.23	72.50 ± 23.18	83.40 ± 23.51	50.00 ± 16.10	68.80 ± 18.78	65.00 ± 27.56	51.33 ± 17.21	68.25 ± 36.77
Morula (n)	**12**	**9**	**13**	**10**	**9**	**7**	**8**	**6**
% out of IVC;	54.00 ± 15.23	61.67 ± 31.89	76.60 ± 32.60	45.00 ± 15.03	68.80 ± 18.78	56.50 ± 31.67	40.17 ± 20.00	63.25 ± 42.77
% out of cleaved	100.00 ± 0.00	83.33 ± 27.97	90.00 ± 22.36	91.75 ± 16.50	100.00 ± 0.00	87.50 ± 25.00	80.50 ± 30.68	87.50 ± 25.00
Compacted Blastocyst (n)	**9**	**6**	**10**	**6**	**4**	**5**	**3**	**3**
% out of IVC	40.00 ± 22.71	31.17 ± 28.50	33.40 ± 42.46	26.50 ± 7.51	27.20 ± 18.57	35.75 ± 26.31	12.50 ± 14.60	30.00 ± 24.49
% out of cleaved	70.83 ± 40.05	44.50 ± 40.46	45.00 ± 51.23	58.25 ± 28.96	46.60 ± 36.16	50.00 ± 40.82	27.67 ± 38.95	37.50 ± 25.00
% out of morula	70.83 ± 40.05	61.17 ± 49.07	50.00 ± 50.00	62.50 ± 25.00	46.60 ± 36.16	62.50 ± 47.87	38.83 ± 49.07	50.00 ± 40.82
Hatched Blastocyst (n)	**4**	**2**	**5**	**1**	**0**	**1**	**0**	**0**
% out of IVC	11.67 ± 20.41	9.67 ± 15.19	18.60 ± 25.59	4.25 ± 8.50	0.00 ± 0.00	8.25 ± 16.50	0.00 ± 0.00	0.00 ± 0.00
% out of cleaved	20.83 ± 33.23	11.00 ± 17.04	28.60 ± 44.04	6.25 ± 12.50	0.00 ± 0.00	12.50 ± 25.00	0.00 ± 0.00	0.00 ± 0.00
% out of morula	20.83 ± 33.23	13.83 ± 22.09	28.60 ± 44.04	6.25 ± 12.50	0.00 ± 0.00	12.50 ± 25.00	0.00 ± 0.00	0.00 ± 0.00
% out of CB	25.00 ± 41.83	16.67 ± 25.82	28.60 ± 44.04	12.50 ± 25.00	0.00 ± 0.00	12.50 ± 25.00	0.00 ± 0.00	0.00 ± 0.00

IVF: *in vitro* fertilization; IVC: *in vitro* culture; CB: compacted blastocyst.
"Oocytes subjected to IVF (n)" indicates the number of oocytes allocated to each treatment group and used for fertilization. "Presumptive zygotes for IVC (n)" represent the number of fertilized oocytes subjected to embryo culture and used as the primary denominator for developmental rate calculations. *A total of 80 and 60 good quality presumptive zygotes from young and old queens, respectively were further evaluated for embryo development under TL system.*
Developmental rates expressed as "% out of IVC" represent the proportion of presumptive zygotes that progressed to each stage. Rates expressed as "% out of cleaved," "% out of morula," or "% out of CB" were calculated relative to the corresponding preceding developmental stage.
Data are presented as mean ± SD; SD = standard deviation.
There were no significant differences between groups.

Using these reference observations, we next explored the potential effects of SRT1720 during IVM. In young queens, the 0.1-μM group showed numerically higher rates of first cleavage and morula formation than the controls, although these differences were not statistically significant (Table 1). A higher proportion of morulae reached this stage before 72 hpi compared to the control group, while no short-cell cycles were observed, consistent with YC (Table 2).

In contrast, the 1-μM concentration showed a more variable distribution of first cleavage timings (Fig. 4) and was associated with slower developmental progression (Fig. 2), with fewer rapid divisions and a higher incidence of shorter cell cycles compared to untreated oocytes (Table 2).

In oocytes from older queens, the 0.1-μM and 1-μM SRT1720 groups showed numerical variation relative to untreated controls; however, no statistically significant differences were detected nor were results fully satisfactory. The 0.1-μM concentration showed developmental results similar to the study reference (YC) up to the late-morula stage, but no hatched blastocysts were observed. The 1-μM concentration showed developmental parameters at the blastocyst stage similar to the study reference and included the sole hatched blastocyst observed in the older queens' experimental groups. This blastocyst originated from an embryo that underwent first cleavage between 20 and 25 hpi. Among the embryos that cleaved within this timeframe, 80% developed into compacted blastocysts. In contrast, the majority of first cleavages in the other groups occurred before 20 hpi (Table 1, Fig. 4). Time-lapse monitoring also documented variation in cell cycle timing among embryos in this group (Tables 1 and 2; Figs. 3 and 4).

4. Discussion

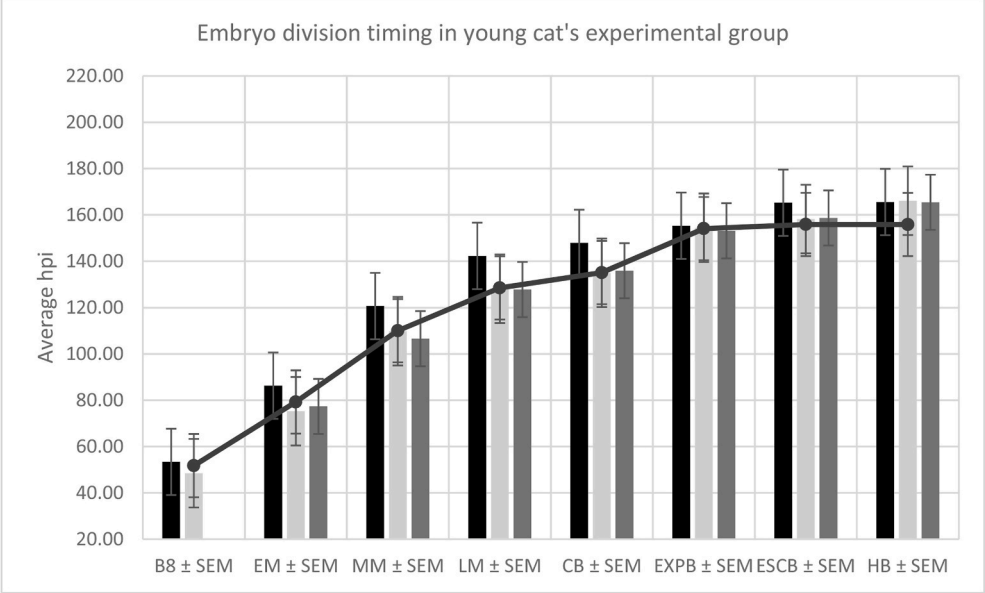
The present study investigated embryo developmental progression and morphokinetic patterns in embryos derived from oocytes of young and aged domestic cats using a time-lapse monitoring system. In addition, the potential influence of SRT1720 supplementation during *in vitro* maturation was evaluated under these conditions. The factorial statistical analysis did not reveal significant effects of maternal age, SRT1720 concentration, or their interaction on the developmental parameters evaluated. Nevertheless, time-lapse monitoring enabled the identification of descriptive developmental patterns that provide insight into early embryonic dynamics in the domestic cat.

Time-lapse systems have become increasingly valuable tools for evaluating embryo development in a non-invasive manner. Continuous imaging allows detailed observation of cleavage dynamics and developmental progression without disturbing the culture environment [56]. In domestic cats, morphokinetic studies remain relatively limited compared with the extensive data available in human assisted reproduction; therefore, descriptive datasets such as the one presented here contribute to the gradual characterization of the kinetics of embryonic development in this species.

4.1. Morphokinetic patterns and cell cycle regulation

In humans, the duration of the cell cycle has been established to range from 10 to 12 h [57], allowing the embryo to undergo two consecutive occurrences of cytokinesis and replicate the entire genome [58]. Embryos exhibiting faster cleavage rates may carry a higher risk of chromosomal abnormalities, as accelerated division may compromise DNA replication fidelity and mitochondrial metabolic homeostasis [59,60], thereby altering the length of cell cycles [61]. This concept suggests that embryo selection based on the timing of the first zygotic cleavage may improve the likelihood of identifying chromosomally balanced embryos [62]. Lemmen et al. [63] highlighted the relevance of time-lapse monitoring in this context. In cats, no such formal correlations have yet been established, though attempts are currently underway [29,30,64].

In the domestic cat, Ochota & Niżański [30] and Kochan et al. [29] noted that embryos dividing rapidly and reaching the morula stage by day 3 post-insemination failed to progress to the blastocyst stage, a finding consistent with the 'quiet embryo' hypothesis proposed by Leese [65], which hypothesize that embryos with minimal metabolic activity and regular cleavage timing are more likely to achieve full developmental competence. In the current study, and in accordance with previous reports [59,65], the control group derived from aged queens exhibited the highest rate of embryos reaching the morula stage before



	B8 ± SEM hpi	B8-16 ± SEM hpi	M16-32 ± SEM hpi	M32-64 ± SEM hpi	CB ± SEM hpi	EXPB ± SEM hpi	ESCB ± SEM hpi	HB ± SEM hpi
Control Young	51.75 ± 2.61	79.25 ± 3.73	110.04 ± 5.09	128.51 ± 5.37	135.10 ± 3.58	154.12 ± 7.52	155.89 ± 8.80	155.87 ± 7.13
1-μM Young	53.39 ± 3.23	86.29 ± 7.04	120.67 ± 7.51	142.32 ± 8.37	147.96 ± 10.44	155.35 ± 8.58	165.27 ± 7.65	165.59 ± 6.54
0.1-μM Young	48.51 ± 3.20	75.24 ± 3.93	109.80 ± 3.27	128.16 ± 2.98	135.01 ± 3.78	154.45 ± 6.09	158.25 ± 3.94	166.14 ± 4.75
0.05-μM Young	(ND)	77.34 ± 6.42	106.58 ± 6.29	127.81 ± 6.88	135.92 ± 5.23	153.15 ± 5.79	158.72 ± 2.90	165.48 ± 0

Fig. 2. Embryo Division Timing (Mean Hours ± SEM) at different SRT1720 concentrations in IVM-treated oocytes from young cats compared to the control group.

Table 2
Rapid divisions and short cell cycles in oocytes from young and old queens across control and SRT1720 treated groups (1-μM, 0.1-μM and 0.05-μM). All embryos reaching the early morula stage before 72 hpi, did progress to blastocyst stage, except in group O0.1. None of short cell cycle divided embryos reached blastocyst stage.

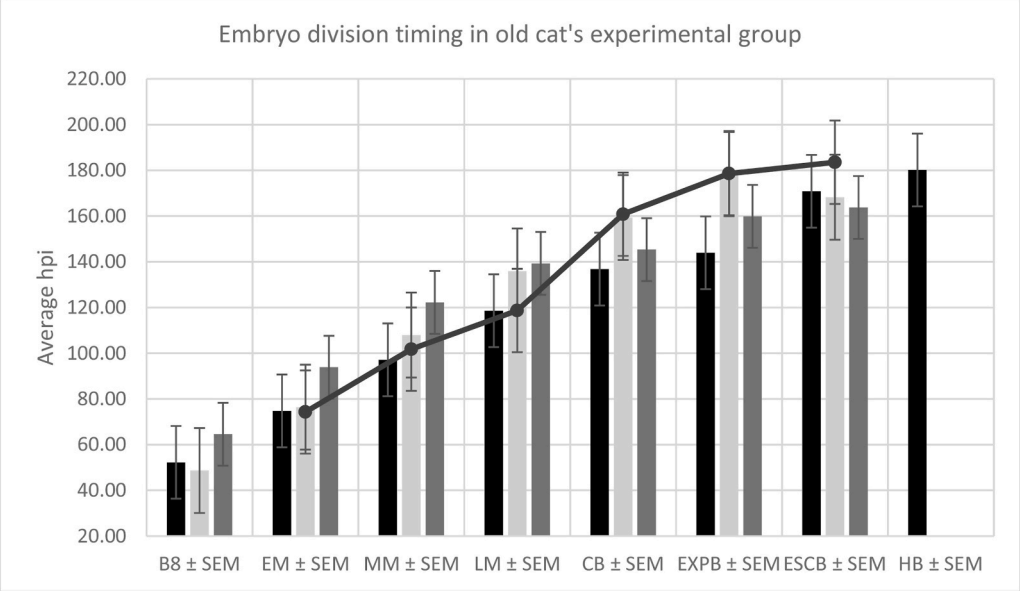
	SRT1720 Treatment							
	Young queens				Old queens			
	C	1-μM	0.1-μM	0.05-μM	C	1-μM	0.1-μM	0.05-μM
IVC (n)	22	17	19	22	14	13	21	12
Total early-morula (n)	12	9	13	10	9	7	8	6
Morula stage <72 hpi								
n (% out of total early-morula)	3 (25%)	2 (22%)	4 (31%)	3 (30%)	4 (44%)	3 (43%)	3 (38%)	0 (0%)
Short Cell cycle (<3h)								
n (% out of total early-morula)	0	1 (11%)	0	2 (20%)	1 (11%)	1 (14%)	0	0

IVC: *in vitro* culture; hpi: hours post-insemination. "IVC (n)" represents the number of presumptive zygotes subjected to *in vitro* culture. "Total early-morula (n)" indicates the number of embryos reaching the early morula stage. Percentages reported for "Morula stage <72 hpi" and "Short cell cycle (<3 h)" were calculated relative to the total number of early-morula embryos within each treatment group.

72 hpi and underwent what Kochan et al. [29] considered short cell cycles as brief as 3 h. However, in contrast to previous studies [29,30], embryos reaching the early morula stage before 72 hpi in the current work progressed to the blastocyst stage (with the exception of group O0.1). None of the embryos exhibiting short cell cycles reached the blastocyst stage.

4.2. Maternal age and developmental dynamics

Embryos derived from aged queens displayed developmental dynamics that differed from those observed in embryos derived from young queens. In particular, embryos from older donors tended to show relatively faster progression through early cleavage stages up to the morula stage, whereas embryos derived from young queens more frequently progressed to later developmental stages such as compacted and hatched blastocysts. Although these observations did not reach



	B8 ± SEM hpi	EM ± SEM hpi	MM ± SEM hpi	LM ± SEM hpi	CB ± SEM hpi	EXPB ± SEM hpi	ESCB ± SEM hpi	HB ± SEM hpi
Control Old	(ND)	74.29 ± 8.21	101.77 ± 10.94	118.70 ± 16.54	160.81 ± 7.90	178.59 ± 9.71	183.53 ± 14.62	0
1-μM Old	52.26 ± 4.66	74.78 ± 4.77	97.11 ± 6.48	118.59 ± 8.01	136.78 ± 14.14	143.93 ± 17.33	170.84 ± 12.69	180.15 ± 0
0.1-μM Old	48.69 ± 2.70	76.41 ± 4.25	107.94 ± 4.88	135.97 ± 5.61	159.38 ± 4.08	178.57 ± 12.00	168.24 ± 0	0
0.05-μM Old	64.55 ± 6.33	93.88 ± 5.65	122.26 ± 9.22	139.32 ± 12.16	145.32 ± 8.84	159.88 ± 9.09	163.77 ± 7.03	0

Fig. 3. Embryo Division Timing (Mean Hours ± SEM) at different SRT1720 concentrations in IVM-treated oocytes from old cats compared to the control group.

statistical significance, they are consistent with previous studies suggesting that maternal age can influence embryo developmental dynamics in several mammalian species [66].

Advanced maternal age (AMA) is associated with reduced mitochondrial function and mitochondrial DNA (mtDNA) copy number in oocytes, leading to diminished ATP production [16–25]. During preimplantation development, the number of mitochondria *per* blastomere decreases due to cytoplasmic dilution and the absence of mitochondrial biogenesis [67,68]. Consequently, blastomeres gradually lose their capacity for ATP production via OXPHOS and show increasing reliance on glycolytic metabolism as the embryo develops to the blastocyst stage [68–70]. At this stage, mtDNA replication resumes primarily in trophectodermal cells, ensuring energy supply for implantation [69,71]. Human studies have demonstrated that AMA impairs mitochondrial function, leading to slower development and reduced morula-to-blastocyst progression [25]. The findings of the present study are consistent with this pattern, as embryos from aged queens displayed delayed developmental progression and decreased transition rates from the morula to the blastocyst stage.

The high energy demands required for blastocoel formation by the TE, which relies on OXPHOS, and for ICM proliferation and maintenance of pluripotency through glycolytic metabolism [69,71], render embryos from aged oocytes particularly vulnerable at these developmental transitions. A recent study in aged mares demonstrated that older animals exhibit lower blastocyst rates due to metabolic limitations in their oocytes; specifically, oocytes from aged mares show reduced capacities for both aerobic and anaerobic metabolism and are unable to compensate for diminished oxidative phosphorylation by upregulating glycolysis, resulting in an overall inability to generate the energy required for critical developmental events [72]. These insights may help explain the reduced blastocyst rate observed in the control group of older queens in the present study, as maternal aging likely compromises the resumption of mitochondrial biogenesis during the morula-to-blastocyst transition.

4.3. *Mitochondrial biogenesis and the premature developmental 'set point'*

Mitochondrial dysfunction observed in embryos from aged mammals results in abnormal resumption of mitochondrial biogenesis driven by the increased energy demands in metabolically stressed embryos [73,74]. A study on early embryogenesis in aged mice reported a premature 'set point', defined as an earlier-than-expected resumption of mtDNA replication. This phenomenon may disrupt the intricate energetic equilibrium and redox status of the embryo, essential for optimal development, and lead to aberrant timing of differentiation events. Subsequently, these embryos may face challenges in establishing a physiological mitochondrial pool at the blastocyst stage [75], consistent with Leese's quiet embryo theory [65]. These findings may explain the observation that, in the present study, embryos from aged queens exhibit accelerated development compared to those from young queens during the early-to late-morula stage, with this trend reversing at the blastocyst stage.

In this study, both 0.1-μM and 1-μM concentrations of SRT1720

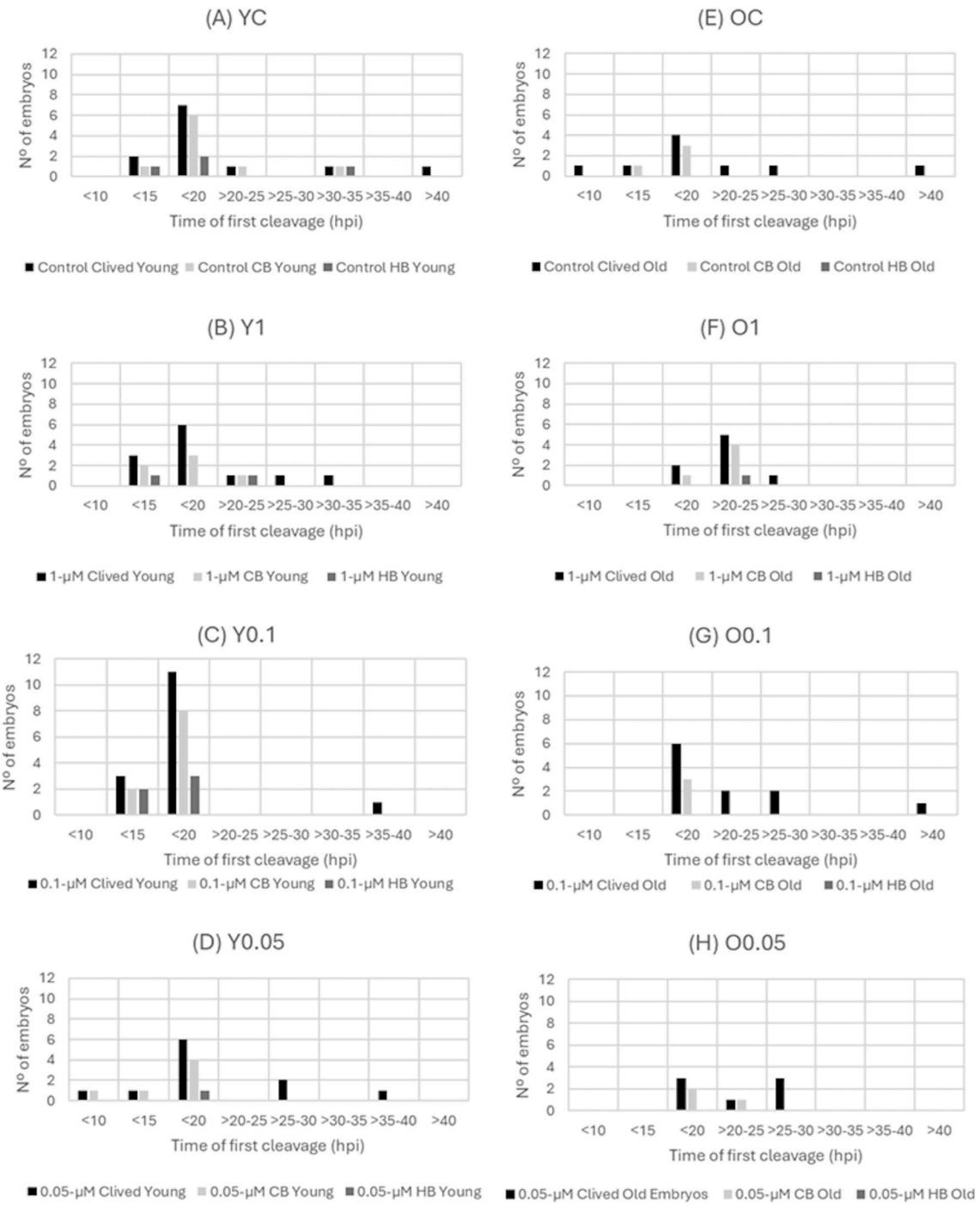


Fig. 4. Relationship between first cleavage timing and blastocyst formation at different SRT1720 concentrations in IVM-treated oocytes from young (n=80) (A–C) and old queens (n=60) (D–G). (A) **Young Control**: 58% of embryos underwent first cleavage within 20 h post-insemination (hpi). Embryos cleaving before 20 hpi were more frequently associated with CB and HB formation rates. (B) **Young 1-µM**: This concentration showed the lowest proportion of early cleavages (<20 hpi), with only 50% of embryos undergoing first cleavage before 20 hpi. Embryos cleaving before 20 hpi were more frequently associated with CB formation, while HB formation occurred either from embryos cleaving before 15 hpi or between 20 and 25 hpi. (C) **Young 0.1-µM**: The highest percentage (73%) of embryos underwent first cleavage before 20 hpi. CB and HB formation were observed predominantly among embryos cleaving before 20 hpi. (D) **Young 0.05-µM**: 55% of embryos cleaved before 20 hpi; (E) **Old Control**: 44% of embryos underwent first cleavage <20 hpi. All CB originated from embryos that cleaved before 20 hpi. (F) **Old 1-µM**: This concentration deviated from the pattern observed in the other seven experimental groups. Most first cleavages (44%) occurred between 20 and 25 hpi, with 25% of embryos cleaving before 20 hpi. Notably, 80% of blastocysts at this concentration originated from embryos cleaving between 20 and 25 hpi, including the sole HB, which developed from a first cleavage at 24.86 hpi. The only HB observed among embryos derived from aged queens originated from this group. (G) **Old 0.1-µM**: 55% of embryos underwent first cleavage <20 hpi. All CB formation occurred in embryos cleaving before 20 hpi. (H) **Old 0.05-µM**: Similar proportions (~43%) of embryos underwent first cleavage before 20 hpi and between >25 and 30 hpi. However, in the latter category, no blastocyst formation was observed.

showed tendencies ($p > 0.05$) that may suggest potential effects on embryo development in oocytes from aged queens.

Oocytes exposed to 0.1-μM showed developmental timings up to the late-morula stage that resembled the study reference. This alignment, observed in 43% of embryos, is considered beneficial as it may help prevent a premature developmental 'set point'. However, despite this temporal synchrony, no hatched blastocysts were formed. These findings suggest that replicating the developmental pace of young oocytes may not be sufficient to restore full developmental competence in oocytes from aged queens.

In contrast, oocytes from old queens exposed to 1-μM showed greater progression to compacted and hatched blastocysts, and a smoother transition from morula to blastocyst compared to the OC group. The timing of CB formation in O1 was similar to that recorded in the study reference, suggesting improved developmental competence. Despite faster early divisions relative to YC – suggesting a premature 'set point', O1 embryos showed comparable CB timings and included the only hatched blastocyst observed among aged cats. Nevertheless, divergence at the expanded blastocyst stage relative to the study reference suggests that developmental competence may remain reduced at later stages.

4.4. Embryo developmental patterns following SRT1720 supplementation in oocytes from aged queens

Embryos in the 1-μM SRT1720 group tended to exhibit more short cell cycles and a modest acceleration in progression to the late-morula stage relative to both control groups. Morula formation before 72 hpi was similar to that in the OC, but hatched blastocysts were observed only in this group. Although accelerated division may contrast with the "quiet embryo" model [65], and has been considered suboptimal by some authors [29,30], under the present conditions, this pattern was accompanied by blastocyst hatching, suggesting that SRT1720 may have supported developmental progression in a subset of embryos derived from aged donors.

The variability in outcomes across embryos from aged queens may reflect differing susceptibilities of oocytes to cellular stress. Older oocytes are generally more susceptible to stress [73,74], which may explain why SRT1720 was associated with more short cell cycles in some embryos while, in others, it may have supported maturation. However, it must be emphasized that the present study did not directly assess mitochondrial activity, oxidative stress markers, or SIRT1-related molecular endpoints; any mechanistic interpretations should accordingly be considered speculative and require further experimental validation.

In contrast, the 0.1-μM SRT1720 concentration in oocytes from aged queens did not present similar effects. Although it maintained regular cleavage timing, avoided premature developmental set points, and achieved morula rates that closely resembled those of the young queens' control group, no clear differences in overall embryo development were observed, with no hatched blastocysts obtained under this condition The 0.05-μM SRT1720 concentration did not induce short cell cycles; however, no significant developmental enhancements were observed, suggesting that this dose was insufficient to produce detectable biological effects under the conditions of the present study.

4.5. Embryo developmental patterns following SRT1720 supplementation in oocytes from young queens

Although pronounced differences in developmental competence were not anticipated in the young queens' experimental groups, the 0.1-μM concentration of SRT1720 was associated with higher cleavage and morula formation compared with the study reference, without apparent short cell cycles. Although descriptive, these observations may support further investigation of this concentration in a larger cohort of young donors to clarify its potential relevance in oocytes considered to have optimal developmental competence [33]1-μM SRT1720 was associated with fewer embryos reaching the morula stage before 72 hpi but with a higher incidence of short cell cycles, suggesting disruption of cell cycle regulation and potential embryonic stress, with fewer morula, blastocyst, and hatched blastocyst formations compared with to untreated oocytes. These results are consistent with the possibility that SRT1720 at 1-μM may have altered the metabolic equilibrium described by Leese's quiet embryo theory [65].

4.6. First cleavage timing as a predictor of developmental outcome

Early cleaving embryos were reported to perform better both during *in vitro* studies or after transfer in humans [76–78] and cattle [79]. In the present study, fewer than 20 hpi was established as the reference time of the first cleavage event. This is consistent with Ochota & Niżański [30], who reported higher blastocyst formation rates in untreated domestic cat oocytes when the first cleavage occurred before 18 hpi. However, it has also been argued that embryos undergoing very early first cleavage (17–18 hpi) exhibited poor development to the blastocyst stage [29].

In the present study, the majority of first divisions occurred before 20 hpi, and higher compacted and hatched blastocyst rates arose from this group – except O1. In that group, the majority of first cleavages occurred between 20 and 25 hpi (44%), compared with only 25% of embryos cleaving before 20 hpi, and the single hatched blastocyst obtained originated from an embryo that underwent its first cleavage at 24.86 hpi. It may therefore be hypothesized that the ideal cleavage window for embryos from aged queens lies between 20 and 25 hpi, and that 1-μM SRT1720 may favor this temporal profile, in accordance with Leese's theory [65], which relates developmental timing to balanced metabolic activity. This time frame aligns with Kochan et al. [29], who reported that untreated domestic cat embryos cleaving between 21 and 24 hpi exhibited the highest developmental potential, and with Klincumhom et al. [64], who found that feline embryos cleaving before 27 hpi showed optimal first cleavage timings, higher blastocyst rates and improved quality relative to embryos with delayed cleavage.

Conversely, in the experiment with young queens, the use of a 1-μM SRT1720 concentration in IVM suggested a deviation from the optimal timing of first cleavage divisions.

4.7. Limitations, sample size, and conservation relevance

This study was conducted between November and March using ovaries obtained opportunistically from free-ranging queens undergoing routine neutering procedures, circumstances that introduced inherent constraints on experimental design. Sourcing donors aged six years or older proved particularly challenging, both in terms of locating eligible animals and obtaining reliable clinical histories. The sampling period coincided with approximately three months of the seasonal anestrus [52], during which the efficiency of *in vitro* oocyte maturation and blastocyst production is known to decline; consistent with previous observations, oocyte quality improved after January [80–82]. These environmental and seasonal variables, inherent to work conducted under non-controlled field conditions, contributed to the outcome variability observed relative to prior controlled murine study investigating SRT1720 supplementation in aged oocytes [33], besides the possible contribution of differences in the species-specific physiology and donor-related heterogeneity. While this variability represents an inherent limitation of field-derived biological material, it also reflects the biological conditions encountered in conservation-oriented reproductive programs.

The absence of statistically significant differences in the factorial analysis must be interpreted in light of these experimental constraints. Access to biological material from aged queens is frequently limited in feline reproductive research, and studies relying on clinical waste tissue routinely contend with unequal and restricted sample sizes across groups. The morphokinetic observations reported here should therefore be regarded as exploratory, intended to identify patterns that may inform the design of future studies with larger cohorts and more

controlled conditions. Additionally, as the study did not directly quantify mitochondrial activity, mtDNA copy number, oxidative stress markers, or SIRT1 molecular targets, the mechanistic interpretations proposed remain inferential and require validation through dedicated molecular and functional approaches.

The decision to prioritize a non-invasive morphokinetic strategy, rather than incorporating direct assessments of mitochondrial function, was deliberate. Isolated assessments of mitochondrial activity typically require embryo removal from time-lapse culture system and are frequently destructive. Continuous, minimal-intervention monitoring preserves embryo integrity throughout development and is particularly relevant in conservation contexts, where minimizing manipulation of genetically valuable material derived from endangered species is paramount. Therefore, mechanism interpretations should be regarded as biologically plausible hypotheses rather than experimentally confirmed pathways and warrant dedicated molecular investigation in future studies.

Despite these limitations, the domestic cat remains an important model for the development and refinement of assisted reproductive technologies with direct applicability to endangered wild felids. Advancing our understanding of embryo developmental dynamics under varying culture and supplementation conditions contributes incrementally to the optimization of reproductive protocols that may ultimately serve conservation programs. Studies of this nature, even when statistically underpowered, continue to generate information of practical value for feline reproductive medicine and for the broader field of wildlife conservation biology.

5. Conclusion

To our knowledge, this is the first study in a felid model to characterize embryo morphokinetics in relation to maternal age and to evaluate SRT1720 supplementation during *in vitro* maturation, assessing both developmental competence and morphokinetic progression. It demonstrated that embryos derived from aged queens display developmental dynamics distinct from those of young queens, consistent with age-related reductions in oocyte competence.

SRT1720 supplementation during *in vitro* maturation did not significantly affect embryo development under the conditions tested. However, time-lapse monitoring enabled the identification of age-dependent developmental patterns and subtle concentration-related differences associated with SRT1720 that may not have been evident through conventional endpoint assessment alone. In young queens, 0.1-μM SRT1720 was associated with numerically higher early developmental rates while maintaining regular division profiles, whereas 1-μM was associated with altered cleavage dynamics. In aged queens, 0.1-μM SRT1720 was associated with the maintenance of reference division timings without clear improvement in later developmental outcomes. In contrast, 1-μM was associated with blastocyst formation, but also with morphokinetic features suggestive of cellular stress, indicating that the effects of SRT1720 may vary with both dose and maternal age.

Taken together, these findings indicate that expecting embryos derived from aged queens to replicate the developmental behavior of embryos from untreated young queens through SIRT1 activation alone may be a challenging objective, and that tailored strategies that address the specific physiological challenges of aged oocytes are required to meaningfully improve their developmental potential.

The morphokinetic observations reported here expand current knowledge of preimplantation development in a seasonally breeding carnivore. These findings may guide future studies using larger cohorts, direct assessments of mitochondrial function, and refined supplementation protocols. Ultimately, advancing our understanding of the factors that govern oocyte competence in the domestic cat has direct implications for the optimization of assisted reproductive technologies applicable to endangered wild felids and for the broader field of wildlife conservation.

Ethical approval

Ethical approval was not required for this study, as it was conducted using surgical waste tissues.

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CRediT authorship contribution statement

Michele Meco: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Rita Payan Carreira:** Conceptualization, Supervision, Writing – review & editing. **Małgorzata Ochota:** Formal analysis, Methodology, Writing – review & editing. **Wojciech Niżański:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing.

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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Data availability

Data is available from the corresponding author on request.

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