Progress toward species-tailored prematuration approaches in carnivores

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Abstract
In the past four decades, the bovine model has been highly informative and inspiring to assisted reproductive technologies (ART) in other species. Most of the recent advances in ART have come from studies in cattle, particularly those unveiling the importance of several processes that must be recapitulated in vitro to ensure the proper development of the oocyte. The maintenance of structural and functional communications between the cumulus cells and the oocyte and a well-orchestrated chromatin remodeling with the gradual silencing of transcriptional activity represent essential processes for the progressive acquisition of oocyte developmental competence. These markers are now considered the milestones of physiological approaches to increase the efficiency of reproductive technologies.
Different in vitro approaches have been proposed. In particular, the so-called "pre-IVM" or "prematuration" is a culture step performed before in vitro maturation (IVM) to support the completion of the oocyte differentiation process. Although these attempts only partially improved the embryo quality and yield, they currently represent a proof of principle that oocytes retrieved from an ovary or an ovarian batch shouldn't be treated as a whole and that tailored approaches can be developed for culturing competent oocytes in several species, including humans.
An advancement in ART's efficiency would be desirable in carnivores, where the success is still limited. Since the progress in reproductive medicine has often come from comparative studies, this review highlights aspects that have been critical in other species and how they may be extended to carnivores.

Keywords
Oocyte, Meiosis, Meiotic arrest, Prematuration, Pre-IVM, Large scale chromatin configuration, Gap junctions, Developmental competence, Embryo, Assisted reproduction, Dog, Cat, Canine, Feline

Introduction

This review aims to extend to carnivores some concepts derived from the experience we have gained in the bovine species using this animal model as a paradigm, given the high efficiency in reproduction technologies in vitro compared to other species.

The quality of the oocytes is fundamental for assisted reproduction outcome, and the maturation of the oocyte represents the first critical limiting step of the in vitro embryo technologies system. In the past three decades, several strategies have been undertaken to overcome some of the intrinsic biological limits of these approaches. However, the percentage of success for in vitro embryo production (IVP) remained stunningly stable and approximately one-third of the oocytes isolated from a given ovary reach the blastocyst stage of embryonic development [1,2].

The development of IVM techniques had its origins in 1935 from Pincus and Enzmann's observations about the spontaneous nature of meiosis when rabbit ova were removed from antral follicles [3]. Thus, in standard IVM protocols, oocytes are collected from antral follicles and cultured in vitro up to the metaphase II (MII) stage, accompanied by the emission of the first polar body and the second programmed meiotic arrest. Nevertheless, when oocytes are routinely collected in pools from antral follicles, the processes needed for the progressive acquisition of full meiotic and developmental competence are not necessarily completed, and a premature engagement in the cell cycle re-entry could compromise the oocyte's ability to be fertilized or develop into embryos or to term.

The bovine oocyte acquires developmental competence when it reaches its final size of approximately 120 µm when the follicle gains 3 mm in diameter [4]. As the follicles grow and undergo selection for dominance, which happens around the 3–8 mm of diameter [5,6], and further on the dominant follicles keeps growing up to about 15 mm before being ovulated [7,8], some events crucial for endowing the oocyte with full developmental capacity occur [9]. These processes were termed "prematuration" or "capacitation" (Fig. 1) [6,10,11].

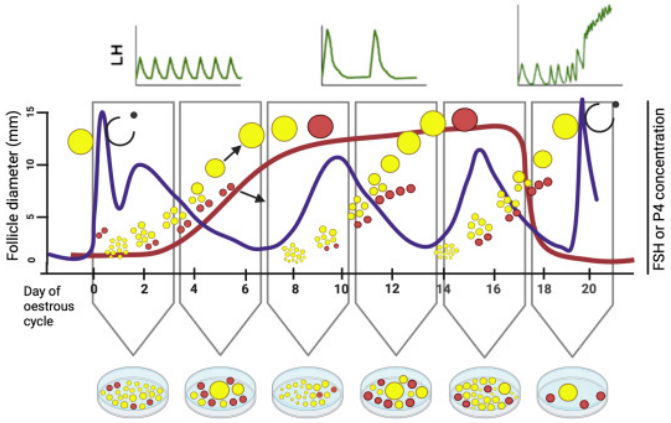

***Figure 1**. Representation of the different populations of oocytes collected from follicles at different stages of development or dismission depending on the estrus cycle phase in the cow. Each vertical tube represents the heterogeneous population of oocytes dropping in the well and submitted to IVP. A schematic depiction of the patterns of hormone secretion and the pattern of growth of ovarian follicles during the estrous cycle in cattle is also presented. Healthy growing follicles are shaded in yellow, and atretic follicles are shaded red. Inspired and modified from Ref. [160].*

Thus, if in the IVP practice only few oocytes isolated from 3 to 8 mm follicles develop into blastocysts that give rise to viable offspring, it is probably because they have not completed the capacitation steps typical of the dominant and preovulatory follicular development [6 3637,10 341]. Furthermore, it must be considered that, when removed from the ovarian environment and cultured, the oocyte population is intrinsically heterogeneous due to the accumulation of antral follicles coming from different follicular waves (Fig. 1). Finally, upon removal from the inhibitory environment of the medium antral follicles, oocytes undergo spontaneous meiotic resumption in the absence of the appropriate follicular stimuli, leading to a loss of synchrony between nuclear and cytoplasmic maturation, overall accounting for a suboptimal developmental competence [12].

Starting from these observations and from almost half a century of knowledge and mastery of the mechanisms that control meiotic arrest and resumption in several mammalian species (Fig. 2), a large body of literature supports the concept of improving the oocyte developmental competence by introducing a culture step before in vitro maturation (IVM) that shall allow the completion of the oocyte differentiation program. The retrieved oocytes are therefore cultured in meiosis-arresting conditions that have been defined as prematuration or pre-IVM.

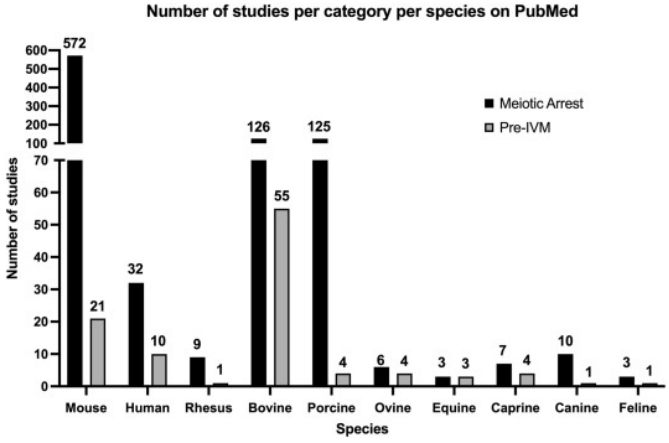

Figure 2*. The number of studies on meiotic arrest and pre-IVM in different mammalian species as available on PubMed. A literature search on PubMed was performed to retrieve the studies discussing pre-maturation and meiotic arrest in different mammalian species. Keywords used for the search were as follows: 'pre-IVM', 'pre-maturation',' capacitation', 'meiotic arrest'. This search was performed on ten mammalian species: Monkey, Mouse, Human, Bovine, Porcine, Ovine, Equine, Caprine, Canine, and Feline, with the respective common and scientific names on the species. The general Boolean query search used was as follows: For pre-maturation studies - ((((((pre-IVM) OR (pre-maturation)) OR (prematuration)) OR (preIVM)) OR (capacitation)) AND (oocyte) NOT (sperm)) AND (∗Species specific terms∗).*

For the meiotic arrest studies - ((meiotic arrest) AND (oocyte)) AND (∗Species specific terms ∗).

Reviews were removed from the search hits, after which manual filtration removed unrelated studies. As indicated, most studies on pre-IVM were conducted on the bovine model, while studies concerning meiotic arrest were conducted mainly on the mouse model. The histogram shows the stark difference in the number of studies conducted on the carnivores compared to other mammals.

Prelude to prematuration: the mastery of the meiotic arrest

The elucidation of the mechanisms that control oocyte meiotic arrest and resumption provided the molecular tools to block or delay spontaneous meiotic resumption [13,14]. Oocyte meiotic arrest depends on high intraooplasmic concentrations of cyclic AMP (cAMP) [15,16] (reviewed in Ref. [13]), a second messenger that controls oocyte and granulosa cell functions [17,18] (Fig. 3). Oocyte cAMP level is maintained by the endogenous adenylate cyclase and constitutively active G-protein-coupled receptors [19,20] but it is also generated by cumulus cells and then transferred into the oocyte through gap junctions [[21], [22], [23]]. Meanwhile, intra-oocyte cAMP concentration is controlled by the activity of the phosphodiesterase 3A (PDE3A), an oocyte-specific enzyme that degrades cAMP into 5'-AMP [24], then converted into ATP during oocyte maturation [25]. In turn, PDE3A activity is inhibited by cyclic guanosine 3',5'-monophosphate (cGMP) [26,27] that is produced in the cumulus cells upon the activation of the guanylyl-cyclase coupled natriuretic peptide receptor type-2 (NPR2) [28] whose activity is induced by its ligand C-type natriuretic peptide (CNP) that is mainly synthesized by mural granulosa cells [29,30]. cGMP is then transported via gap junctions [31] to the oocyte, thus inhibiting PDE3A and contributing to the maintenance of meiotic arrest [26,27].

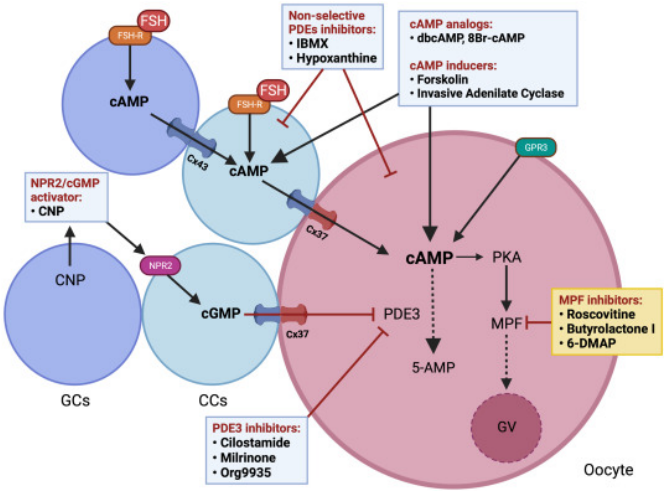

***Figure 3**. A simplified diagram illustrating the pathways involved in maintaining mammalian oocyte meiotically arrested at the GV stage, with the participation of FSH/FHSR, CNP/NPR2, and GPR3. The commonly in vitro used meiosis arresting agents and their targets are in the boxes. In the cumulus cell, CNP activates its cognate receptor NPR2, which converts GTP into cGMP, then cGMP diffuses into the oocyte through gap junctions and inhibits PDE3 activity, blocking the degradation of intraoocyte cAMP. The high level of cAMP suppresses the maturation-promoting factor (MPF) activity maintaining the oocyte arrested at the GV stage. Removing the oocyte from the follicle induces a decrease in cyclic AMP and cGMP intraoocyte levels leading to GV breakdown and meiotic resumption. Molecules used to maintain oocyte meiotic arrest in vitro following removal from the follicle act upregulating oocyte cAMP level or suppressing MPF activity.*

For more than 50 years, many pharmacological and physiological agents have been used to modulate the oocyte cAMP concentration to temporally control the oocyte's meiotic arrest and resumption [32]. The cAMP concentration has been controlled using a broad spectrum of unspecific, such as 3-isobutyl 1-methylxanthine (IBMX), or specific PDE3A inhibitors, such as milrinone, cilostamide, or Org9935; cAMP levels have also been manipulated by activators of adenylate cyclase (forskolin, invasive Adenylate Cyclase, iAC), cAMP analogs (dbcAMP, 8-Br-cAMP,

Rp-cAMPS) or by a combination of these agents [32]. Several groups have shown that delaying meiotic resumption positively affects oocyte embryonic developmental competence. Notably, the intracellular cAMP concentration affects the functional coupling between oocyte and cumulus cells so that a decrease in the oocyte cAMP determines a drop in gap junction-mediated intercellular communications [33,34] while treatments that sustained an appropriate intraooplasmic cAMP level prevented the loss of gap junction functionality and promoted an increase in oocyte developmental competence [[35], [36], [37], [38], [39], [40], [41], [42], [43], [44], [45], [46], [47], [48]]. Over the years it became increasingly evident that the preservation of the cAMP concentration during prematuration is a main requirement to promote regular chromatin transition (see below) thus supporting oocyte differentiation [40,46,47,[49], [50], [51]] and the increase in embryo yield and/or quality [46,47,[52], [53], [54], [55], [56]].

It is possible to prevent meiotic resumption also acting downstream the cAMP transduction pathway, directly inhibiting the M-phase promoting factor (MPF), a heterodimer consisting of a kinase, CDK1, and a regulatory partner, cyclin B. This complex is involved in the regulation of the G2/M cell cycle transition of all eukaryotic cells (Fig. 3) and it is controlled upstream by the cAMP-mediated protein kinase A (PKA) activity, which contributes to the oocyte meiotic arrest by inhibiting CDK1 [57]. MPF activation is a key point for meiotic resumption in oocytes that corresponds to a G2/M transition [57,58].

Multiple pharmacological approaches that interfere with MPF activity maintain mammalian oocytes in meiotic arrest. Cell permeable and selective CDK1/cyclin B kinase inhibitors, such as butyrolactone-I [59] and roscovitine [60] have received more attention. Butyrolactone-I has been shown to reversibly inhibit meiotic resumption in bovine [57,[61], [62], [63], [64], [65],66,67,68,73] and pig [69] oocytes for 24 h without affecting the subsequent development to the blastocyst stage. Similarly, roscovitine was effective in reversibly maintaining the oocyte in meiotic arrest in cow [70,71] and pig [72], and a combination of both substances did not cause detrimental effects on blastocyst development [63] and subsequent early stages of organogenesis [65]. However, most studies documented no significant improvements in embryonic developmental competence when oocytes were arrested with MPF inhibitors before IVM compared to the direct IVM culture. Nonetheless, some studies reported that butyrolactone and roscovitine induced modifications in the oocytes at the ultrastructural level [73], and whether these modifications are compatible with normal gestation and live births is still not proven.

Based on current evidence from the literature, the control of cAMP levels is the favorite since they have proven to be effective in premature approaches in different species.

Markers in prematuration approaches

The key to a successful in vitro pre-maturation relies on the selection, using non-invasive markers, of a homogeneous population of gametes that can benefit from the prolonged culture itself. At the same time, the quality of oocytes at advanced stages of differentiation might even be compromised by prolonged in vitro culture [74].

The concept that the fraction of fully-grown oocytes that would most benefit from a pre-maturation treatment are those showing a not fully compacted chromatin enclosed within the germinal vesicle (GV) was elaborated in 2007, when our group, stemming from earlier studies in the mouse model [75,76], started by considering the large-scale chromatin organization as a marker of oocyte differentiation in GV stage bovine oocytes [77]. However, the hypothesis was experimentally proved almost ten years later [74] after intensive research in both cattle and other species [46,47,50,52,[78], [79], [80], [81], [82]].

This hypothesis was supported by previous experimental observations published around the 2000s. One of these studies had suggested that pre-maturation may be beneficial to oocytes in

their growing phase [83]. Other studies in bovine had demonstrated that the morphology of oocytes belonging to medium antral follicles was negatively affected by pharmacological pre-treatment compared to those isolated from smaller follicles [84]. In addition, findings in the mouse model indicated that experimentally extending the interval between transcriptional inactivation and meiotic resumption may be deleterious to subsequent embryonic development [85].

Studying the rearrangements occurring within the nucleus of the immature oocyte accompanying its growth and differentiation has been crucial for developing a tailored pre-maturation system. It must be addressed here that the 'large-scale chromatin configuration changes' to which we refer are those events acting to remodel wide portions of the chromatin mass and that can be visualized under a fluorescent microscope after DNA staining, rather than those operating locally on specific loci [86]. Morphologically, indeed, during meiotic arrest, and particularly during the oocyte growth phase leading to the formation of the fully-grown and differentiated oocytes, the chromosomes lose their individuality as well as their characteristic appearance, and form a loose chromatin mass [87,88], which undergoes profound and dynamic rearrangements in the GV before meiotic resumption. Typically, chromatin appears in a less condensed state, in which it is dispersed throughout the nucleoplasm and then compacts, concentrating in a small area of the nucleoplasm, often in close association with the inactive nucleolus [86]. This structure has been previously described as the karyosome or karyosphere [87,[89], [90], [91], [92]].

Multiple studies in different mammalian species have clearly indicated that chromatin configuration within the GV is a reliable marker of oocyte differentiation at the time of isolation from the follicular environment and as long as the oocyte is meiotically arrested [86]. However, for the sake of simplicity, in the following part of this paragraph, we will only summarize the studies in the bovine model.

In bovine oocytes, four stages of chromatin configurations have been described: the GV0 stage is characterized by filamentous chromatin diffuse in the whole nuclear area; the GV1 and GV2 configurations represent early (GV1) and intermediate (GV2) stages of chromatin compaction, while the GV3 is the stage where the maximum level of compaction is reached with chromatin organized into a single clump. While GV0 oocytes are typically enclosed in small antral follicles of 0.5–2 mm in diameter, the other three stages - considered "fully grown oocytes" - are found within follicles at more advanced stages of antrum development. Unfortunately, we never had the chance to observe the oocyte chromatin enclosed within the dominant follicle. In addition, while a certain level of correlation between the oocyte diameter and the chromatin configuration has been documented [77], a more precise correlation between increased levels of chromatin compaction and follicle size could not be confirmed for GV1, GV2, and GV3 oocytes, as their distribution did not change in follicles of 2–4, 4–6 and >6 mm in diameter [74]. As such, oocytes collected from middle antral follicles represent a heterogeneous population of oocytes roughly composed of one-third GV1, one third GV2, and one third GV3 oocytes, with distribution often depending on the "batch" of ovaries collected at the abattoir.

Other than the assessment of the diameter and the distribution, the first experimental confirmation of a correlation between chromatin configuration and oocyte differentiation came from experiments in which we divided living oocytes according to their chromatin configuration using Hoechst 33342 staining before culturing them in a standard IVM, IVF and embryo culture system [77]. These experiments indicated that while the acquisition of the meiotic competence is timely associated with the appearance of early signs of chromatin compaction (GV1), the embryonic developmental competence is acquired before the highest level of chromatin compaction is reached [77], with GV1 oocytes developing to the blastocyst stage at a lower percentage than GV2 and GV3 oocytes [77]. Moreover, while GV0 oocytes are transcriptionally

active and show lower levels of global DNA methylation, GV1, GV2, and GV3 oocytes are progressively silenced, with an abrupt decrease of transcriptional levels observed during the GV0 to GV1 transition, as well as with higher levels of global DNA methylation [50,80,93,94]. Considering other epigenetic marks, such as histone modifications, our data indicated that they might be acquired in a stage-specific manner, at least when considering Histone 4 acetylation at some specific lysine (K) residues. For instance, acetylation of H4K12 is already established in GV1 oocytes, while acetylation levels of H4K5 are higher in GV2 compared to GV1 oocytes [95].

Electron microscopy also revealed distinctive features of the cytoplasmic organelles in each GV stage, which are, in turn, crucial for the oocyte to achieve meiotic and developmental competence [10,80,96]. The ultrastructure of the GV0 oocytes was typical of growing oocytes. In contrast, characteristic features denoting completion of the oocyte growth phase, such as undulation of the nuclear envelope and reduction in size of the Golgi complex in the oocyte cortex, were found associated with high levels of chromatin compaction in oocytes with GV2 and GV3 configurations. Notably such features closely resemble the ones observed along the process of "oocyte capacitation" [10] that has been described for the dominant follicle, during preovulatory development before the LH surge [10,97]. Some features of early atresia, such as initial degeneration of the cortical granules, were found in GV3 oocytes [80].

Not entirely surprisingly, the assessment of the intercellular coupling between the oocyte and the surrounding cumulus cells by microinjection of a fluorescent dye in the oocyte cytoplasm and, eventually, its diffusion into the surrounding cells through open gap junctions (GJ) revealed that while the high majority of GV0 oocytes are fully coupled with their surrounding cells, this interplay is progressively reduced from GV1 to GV3 oocytes, with a percentage of GV3 oocytes with closed GJ mediated communication as high as 64% [77]. The latter is a condition that, together with other the cell-to-cell contact loss, is related to the tendency to apoptosis of the cumulus cells [98].

Molecular confirmation that oocytes with different patterns of large-scale chromatin configuration are indeed different and that GV3 oocytes are predisposed to atresia came from transcriptomic studies of both the oocytes with varying chromatin configurations [78] and their surrounding cumulus cells [74]. Specifically, in silico analysis of differentially expressed genes in the cumulus cells of GV0, GV1, GV2, and GV3 oocytes revealed that, when compared to GV0, apoptosis is inhibited in the cumulus cells of GV1 and GV2 oocytes (although at a lower extent), while it is favorited in cumulus cells of GV3 oocytes [74].

It is important to underline that features associated with oocyte degeneration in subordinate follicles, usually referred to as "pseudo-maturation" [97], also show similarities to the process seen during capacitation in dominant follicles [97,99]. Therefore, and since in vitro developmental competence seems to be improved by low levels of atresia [9,[99], [100], [101], [102], [103], [104], [105]], it is not surprising that some features of early atresia are also typical of GV3 oocytes. Overall, these observations seem to suggest that oocytes with the highest level of chromatin compaction represent that proportion of gametes that had reached a high developmental capability during follicular growth and were undergoing early events of atresia at the time of collection [77]. Whether the higher competence to produce blastocyst in vitro is also accompanied by higher pregnancy and calving rates still needs to be proved.

Altogether these data were fundamental for the development of the concept of a tailored prematuration system to overcome the limitation imposed by the heterogeneity of the population of bovine fully grown oocytes isolated from naturally – not synchronized – cycling females, which is, in our opinion, the leading cause of the limited success of pre-IVM so far.

Thus, ideally, GV0 oocytes would require a prolonged in vitro culture to complete transcription and reach the fully-grown state; GV1 oocytes are those who might benefit from pre-

IVM, and GV2 is the 'gold standard', ready to be in vitro matured, while prematuration would not be required, or even deleterious, for GV3 oocytes.

The experimental proof of this hypothesis would require a non-invasive marker to univocally select oocytes according to their chromatin configuration and monitor their remodeling during culture. Unfortunately, such a marker, or combination of markers, is not yet available, even though intensive research is in place in several species to use the transcriptome profile of the cumulus cells to predict the developmental competence (i.e., the level of differentiation) of the enclosed oocyte [[106], [107], [108]]. The goal would be to collect a small biopsy of the cumulus oophorus before IVM, without perturbing oocyte viability, and evaluate expression of genes used as markers to predict the corresponding oocyte's characteristics.

Nevertheless, we indirectly proved the above hypothesis in a proof-of-concept experiment by taking advantage of pioneering studies in bovine [100]. In their studies, Patrick Blondin and Marc-André Sirard demonstrated that COCs with different morphological characteristics have different in vitro developmental potential. Precisely, three morphologically healthy classes were identified, of which the class 1 COC presented a homogeneous ooplasm and absence of expansion of outer layer cumulus cells; class 2 COC was characterized by slight granulation of the ooplasm and mostly compact layers cumulus cells; class 3 COC had highly granulated ooplasm and few outer layers cumulus cells showing expansion. Notably, class 3 showed the highest in vitro developmental competence [100]. In our studies, when we isolated the COCs from middle antral follicles and divided them into the three above mentioned classes, class 1 COCs were the only ones in which oocytes with GV1 chromatin configuration could be found, while they were almost absent in class 2 and 3 COCs [74]. Furthermore, Brilliant Cresyl Blue (BCB) staining of class 1, 2, and 3 COCs indicated that class 1 COCs are at an earlier stage of differentiation when compared with class 2 and 3 COCs, giving additional (indirect) confirmation that chromatin compaction is associated with oocyte (and accompanying CC) differentiation [74]. Thus, we identified a GV1-enriched subpopulation of oocytes (class 1) and a GV2/GV3-enriched subpopulation (class 2/3) based on the morphological criteria of the COCs.

When subjected to standard IVP procedures (with regular IVM protocol), our data confirmed previous evidence that class 1 COC showed a lower developmental competence when compared to the class 2/3 group. As expected, the group composed of the mix of class 1, 2, and 3 COC, which correspond to the group of COCs commonly used in IVP protocols, showed intermediate values between class 1 and 2/3 COC. On the other hand, pre-IVM treatment significantly increased the developmental capability of class 1 COCs and did not affect the mixed group, while it reduced the developmental competence of class 2/3 COC [74]. These data confirmed our hypothesis that the fraction of fully-grown oocytes that mainly benefit from a pre-maturation treatment are the GV1 oocytes and set the stage for further analysis that more univocally allow the identification of oocytes with different chromatin configuration, which would ultimately allow the optimization of 'tailored' culture system for each stage.

To conclude this paragraph, it is crucial to warn the readers that attempts to manipulate in vitro large-scale chromatin configuration must be performed cautiously. Chromatin remodeling must occur 'physiologically', and pharmacological treatments forcing chromatin abruptly into a high condensed state may not necessarily be beneficial to the oocyte competence [109]. Therefore, the design of pre-maturation strategies must consider that chromatin condensation and spatial reorganization should occur gradually and orderly, recapitulating the physiological process in vivo. For example, maintaining a proper functional coupling between oocyte and cumulus seems crucial in sustaining an orderly chromatin condensation in vitro [50]. Thus, if the coupling is prematurely interrupted - i.e., when oocytes have not yet acquired complete competence as they are still committed to accumulating transcripts and other molecules - unexpected chromatin

condensation can be triggered, thus preventing proper and gradual differentiation of large-scale chromatin configuration and function. Because of all given considerations, knowledge of the molecular mechanism(s) leading the oocyte to remodel its chromatin configuration under physiological conditions will help ART.

State of the art in prematuration approaches in carnivores

Domestic carnivores are among the species that could benefit the most from ameliorative approaches in IVP, whose efficiency is still limited [110,111]. In cats, the oocyte maturation rate reaches 60%, and upon IVF cleavage rate can go up to 60%, with half of the cleaved embryos reaching the blastocyst stage [112]. In dogs, due to the peculiar reproductive physiology, only a small percentage of oocytes can mature in vitro (10–20% from anestrous ovaries, up to 30% from preovulatory follicles [113]). Upon IVF, which is limited by fertilization failure and polyspermy, only around 10% of the oocytes show regular fertilization, and embryo development is often blocked at the 4- to 8-cell stage [113]. Blastocysts are rare, and no live births have been obtained from procedures carried out entirely in vitro.

In both species, oocytes are usually obtained from isolated gonads by mincing the ovarian cortex in the collection medium, and oocytes are selected by morphological grading based on their size, shape, cytoplasm, and cumulus cells [[114], [115], [116]].

In vivo physiology differs between cats and dogs. That of dogs strikingly differs from other mammals.

The domestic cat is a seasonally polyestrous species. Cats are polytocous, and ovulation can be spontaneous or induced [117]. Like most mammalian species, oocytes are ovulated at the MII stage [110]. Season and ovary stage can influence in vitro outcomes of oocytes collected from isolated gonads, with spring-summer and luteal or intermediate ovaries (1- to 2-mm follicles on at least one ovary) being the most favorable combination [118].

On the other hand, dogs are well-known for ovulating immature, GV stage oocytes, probably their most particular reproductive feature. Polytocous and spontaneous ovulators, dogs are non-seasonal monoestrous with long and variable inter-estrus intervals (5–12 months) [119]. After ovulation, the oocytes need at least two days in the oviduct, with high circulating progesterone concentrations, to reach the MII stage and be ready to be fertilized [119,120]. IVM of oocytes collected from isolated gonads is still challenging. No agreement on the length of culture and microenvironment properties has been found. Information on the influence of the estrous stage of the bitch on IVM rates is contradictory [121], but it seems that oocytes retrieved during anestrus, the most prolonged phase, with no follicular activity, have a low competence for meiosis, even if cultured in vivo [122]. The low competence could be linked to the impaired GJ functionality between oocyte and cumulus oophorus cells during the anestrous [123]. By contrast, almost 90% of COCs retrieved from late proestrus ovaries showed open GJs at collection, matched by higher maturation rates [123]. These observations made by lucifer yellow dye functional coupling assay were also recently confirmed by the analysis of connexin 37 and 43 expression patterns, and overall, they indicate a pivotal role of cell-to-cell communications during follicle growth and estrous cycle [124].

The ‘physiological oddities’ could be one of the reasons for the limited efficiency of IVP systems in carnivores, especially in dogs, together with the fact that studies on oocyte biology and intracellular mechanisms are still scarce compared to other species. As mentioned above, in other mammals, specific events were identified (e.g., (un)coupling between oocytes and cumulus cells, chromatin remodeling, silencing of transcriptional activity) and correlated with the acquisition of oocyte developmental competence. This knowledge is indeed poorer in domestic carnivores; consequently, prematuration has also been barely investigated in cats and dogs. Among the few

studies available, some aimed to evaluate the efficacy of the meiotic arrest of oocytes, while others used a prematuration approach (i.e. combining a culture step supporting the meiotic arrest in the absence of any stimulation, followed by IVM) (Table 1).

***Table 1**. Studies on meiotic arrest and pre-IVM in carnivores*

Species	Treatment	Effect on GV arrest	Effect on MII rate	Effect on blastocyst rate	Reference
Cat	Roscovitine	Positive	No effect	negative	[125]
Cat	Forskolin Isobutyl methylxanthine (IBMX)	No effect Positive in spontaneous or induced maturatio	N/A No effect	N/A No effect	[126]
Cat	C-type natriuretic peptide (CNP)	Positive at 100 nM and 12 h of incubation	N/A	N/A	[127]
Dog	cAMP analog	Positive in a dose dependent manner	N/A	N/A	[131]
Dog	cAMP analog and P4	No effect	No effect	N/A	[128]
Dog	cAMP analog Roscovitine	Positive No effect	No effect No effect	N/A N/A	[130]
Dog	eCG & E2 Butyrolactone I Roscovitine eCG & E2+ Roscovitine	Positive No effect No effect Positive	N/A No effect No effect Positive	N/A N/A N/A N/A	[129]

In the domestic cat, the MPF inhibitor roscovitine was the first substance to be tested in a prematuration protocol [125]. Its effects were investigated on excellent quality (grade I) cumulus-oocyte complexes (COCs), and on grades II and III to distinguish different oocyte populations and in an attempt to improve the developmental competence of poorer quality COCs. The ability of roscovitine to maintain the GV arrest was confirmed for both oocyte categories. However, while for grade I COCs it was dose-dependent and increasing concentration of the blocker (until 100 µM) also allowed decreasing degeneration rates, for grade II-III oocytes, the concentration of roscovitine (12.5–100 µM) did not influence the proportion of GV-arrested or degenerated oocytes [125]. However, these encouraging results were insufficient to design a successful prematuration approach. After 24 h of incubation with roscovitine, followed by IVM, the proportion of matured oocytes did not significantly differ from the untreated control for grade I and grade II-III oocytes [125]. The same treatment also decreased developmental competence, and oocytes undergoing prematuration with roscovitine showed significantly lower cleavage and blastocyst rates than the control [125].

More recently, isobutyl methylxanthine (IBMX) and forskolin were used to regulate cAMP levels to control meiosis block in a “spontaneous” and “induced” maturation in cat oocytes [126]. However, since acting on cAMP levels with two different mechanisms (Fig. 3), the administration of IBMX and forskolin have produced contrasting results. Forskolin was ineffective in maintaining GV-

arrest of cat oocytes, allowing meiosis resumption [126]. The use of IBMX, instead, was able to keep the oocytes in the GV stage, both in spontaneous maturation conditions (i.e., absence of any maturation-promoting stimuli) and in induced maturation conditions (i.e., presence of equine chorionic gonadotropin - eCG and epidermal growth factor - EGF) [126]. Nevertheless, no improvements were observed, and meiotic arrest with IBMX followed by IVM was just as good as standard IVM regarding MII and embryonic developmental rates [126].

Following a different trend and using the physiological inhibitor CNP, another research group attempted to control cyclic nucleotides and oocyte meiosis in the domestic cat [127]. Similar to roscovitine, CNP addition helped reduce meiosis resumption, maintained the GV arrest and decreased the degeneration rates of incubated oocytes [127]. In addition, this result was associated with the retention of cGMP in CNP-treated oocytes, which showed, after 24 h of incubation, cGMP levels similar to those of oocytes at the time of collection and significantly higher than oocytes incubated without CNP. Since then, to our knowledge, no other attempts have been made to create a prematuration system for feline oocytes. The design of a physiological and efficient feline prematuration system deserves further investigation to assess the species-specificity of inhibitors (e.g., the detrimental effect of roscovitine, meiotic resumption with forskolin) and peculiar oocyte biology in cats (e.g., lower spontaneous maturation rate than other species, PDEs activity, etc.).

In dogs, instead, probably due to the complexities in developing efficient IVM systems, some other “prematuration-like” approaches were tested, even earlier than in the cat. Not being a conceptually genuine prematuration step, these culture conditions have been usually defined in other ways, such as “Bi-phasic system” or “Combinatorial IVM” [128,129]. Different meiosis inhibitors have been tested (e.g., dibutyryl cyclic adenosine monophosphate (dbcAMP), butyrolactone, and roscovitine). However, while they could maintain oocytes in meiotic arrest to different extents, no significant improvements were observed upon IVM. Furthermore, treatment with meiosis-inhibiting compounds was often carried out with the simultaneous addition of stimulating molecules (e.g., hormones, serum, EGF). The rationale for this choice may be linked to the peculiar physiology of the dog. Hormonal stimulation during the immature oocyte stage could resemble the in vivo conditions, where LH peaks before ovulation, high estrogens and FSH are present, progesterone production is initiated in the follicle well before ovulation and at higher concentrations than those the oocytes will find in the oviduct [[128], [129], [130]].

Previous reports indicate that meiosis resumption of dog oocytes can be controlled with dbcAMP, a synthetic cAMP analog. Arrest at the GV stage was obtained [131] even if an inconsistent ability to block meiosis resumption in different batches of ovaries was reported [130]. The effect of dbcAMP was reversible, and following its removal and IVM, oocytes could resume meiosis. No beneficial effects were observed on maturation rates, though [130], not even when different concentrations of progesterone (0–2000 ng/ml) were tested both in inhibition medium and maturation medium to better mimic the physiological changes in progesterone levels, nor when oocytes were treated separately based on the ovary stage (proestrus/dioestrus) [128].

Exploiting the approach of MPF inhibition led to similar results. Different concentrations (0–100 µM) and exposure times (24–48 h) to butyrolactone I and roscovitine were tested, but the outcomes were similar between control and treated oocytes in terms of meiosis resumption [129] or MII rates [130].

In a previous study, Hanna and collaborators aimed to develop an efficient system to keep the oocyte meiotic arrest by recreating the hormonal follicular conditions based on the hypothesis that a drop in hormone concentration stimulates meiosis resumption [129]. Estradiol and eCG were used after observing that in vivo-matured canine oocytes obtained from bitches treated with eCG had a higher GV rate than oocytes collected from bitches that were not exposed to eCG [129].

Neither estradiol (2 ng/ml) nor eCG (10 IU/ml) alone had a significant effect on GV arrest, but their combination was effective, and it decreased the meiosis resumption rates following 72 h of incubation [129]. Based on this finding, these hormones were used in combination with roscovitine for 48 h of incubation, resulting in higher GV rates compared to the control or roscovitine-only treated oocytes [129]. After IVM, meiosis resumption rates were also higher with this combination, although not significantly compared to those obtained with roscovitine alone [129], representing the best result achieved so far.

Pre-incubation with roscovitine alone for 15–17 h, followed by a short incubation with eCG before IVM, was also studied because brief exposure to a low dose of gonadotropins before IVM was beneficial for MII rates in other species, but the same effect was not observed in the dog, regardless of eCG exposure time or IVM length [130]. Unfortunately, quantification of cAMP following the use of meiosis inhibitors or other molecular investigations has not been performed in dog oocytes. This data would give us more information to develop customized prematuration (or enhanced IVM) systems for this species, whose reproductive peculiarities are evident.

Altogether, these findings in cat and dog oocytes indicate, once again, some differences in domestic carnivores compared to other species and the need for species-specific investigations. Understanding why most immature oocytes do not spontaneously resume meiosis in vitro after removal from the follicle [128] could be a good start. In addition, large variability between replicates is often observed [130] and more investigations should be performed on the unsolved basic issues of carnivore IVM, such as the influence of ovary stage and season, for which we still do not have a clear answer, and that could be confounding factors in prematuration studies. Further studies on prematuration time and the actions of the inhibitors on the somatic compartment of the COCs would also be necessary.

A hypothetical tailored approach

Bringing together the considerations made thus far on pre-IVM approaches in mammals, the peculiarities of carnivore's reproductive physiology, and the available knowledge of basic oocyte biology in cats and dogs, we will analyze in this paragraph the possibility of designing pre-IVM approaches tailored to these species.

As already mentioned, observing that the GV chromatin compaction was associated to the progressive acquisition of meiotic and developmental competence prompted investigations to capture the changes in the GV chromatin status in several mammals, including cats and dogs. Quite surprisingly, though, significant changes in the chromatin landscape were not observed in cat oocytes, where the chromatin remained dispersed in a reticular configuration throughout the nucleus even in fully grown, transcriptionally silent oocytes retrieved from medium and large antral follicles [109]. While this lack of main events of chromatin compaction remains unexplained, other features of oocyte final growth and differentiation were maintained. Indeed, cat oocytes sequentially acquired meiotic and developmental competence at variable degrees in the transition from early to small antral follicles and from small to medium antral, respectively. Furthermore, this last transition was accompanied at a structural level by the loss of functional nucleolar-like bodies [109].

Despite the apparent homogeneity of cat oocytes retrieved from medium-large antral follicles in terms of large-scale chromatin remodeling, morphological grading allowed to sub-classify feline COCs into three categories. Interestingly, these categories also showed different diameters (115.7 ± 4.2, 116.2 ± 2.8, and 109.2 ± 5.1 for grades 1, 2, and 3, respectively) and developmental competence progressively decreasing from grade 1 to grade 3 [109]. Indeed, in most studies, grade 3 oocytes are discarded due to their lower quality and performance in vitro and are generally perceived as a 'degenerating' category. However, an alternative interpretation

might be that grade 3 oocytes have not yet accomplished the last steps of the growth and differentiation program. On this line of reasoning, it might be worth noticing that the relatively small difference in linear diameter – roughly 6–7 µm – accounts for a 15–20% smaller sphere when converted in volume.

Suppose the hypothesis of grade 3 oocytes being 'not fully grown' will be experimentally proven, in that case, the possibility of clustering COCs retrieved from medium-large antral follicles using a simple, non-invasive, morphological analysis may allow to develop tailored pre-IVM approaches in cats similar to what was previously reported and extensively discussed in paragraph 3 for cows [74].

A similar attempt to sub-classifying feline COCs was made by Sananmuang and colleagues [125], separating grade 1 from grade 2–3 oocytes and testing the effects of increasing concentrations of the MPF blocker roscovitine, as discussed above. In their hands, lower levels of the inhibitor were sufficient to block meiotic resumption in grade 2–3 compared to grade 1, and a roscovitine-based pre-IVM culture improved, at least in part, the meiotic competence of grade 2–3 oocytes, while being mainly detrimental to grade 1. If these results seem to sustain the hypothesis that grade 3 oocytes represent a class of gametes that are not fully differentiated and might benefit from prematuration, a 'rescue' of the developmental competence was not obtained. However, other considerations should be taken into account to interpret these data. For instance, targeting MPF to inhibit meiosis resumption consistently gave lower results in pre-IVM settings in most mammals than targeting the cyclic nucleotide levels [82].

Another non-invasive parameter to identify sub-populations of COCs that might or might not benefit from an extra culture step before IVM may be represented by the ability to metabolize the BCB dye as an indirect measurement of the glucose-6-phosphate dehydrogenase (G6PDH) activity. Generally speaking, G6PDH is more active in growing oocytes and reduces the uptaken cytoplasmic BCB to a colorless compound, while fully-grown oocytes will show a blue/violet staining due to the low G6PDH activity [132]. In cats, this assay successfully identified a COC class, the BCB+, that performs substantially better in IVM-IVF-IVC settings compared to the BCB- [133], also in the seasonal anestrous [134]. Conversely, in dogs, BCB staining was not associated with morphological or biochemical parameters of oocyte quality [135].

In cats, it remains to be experimentally determined whether the BCB- class represents a quota of growing oocytes that can be successfully submitted to prematuration in vitro. Although this conclusion may seem obvious given the premises of the assay and the findings in other mammals, ultrastructural analyses showing the migration of the GV at the periphery, enlarged perivitelline space, and elongated mitochondria might suggest otherwise. Therefore, experiments aimed at understanding the origin of cat BCB- COCs need to be performed to fill this gap.

If the possibility of successfully stratifying the population of feline COCs according to their cultural needs is a positive element for the application of tailored pre-IVM approaches, a consideration that must be done relates to the extent to which the GJs between the somatic and germline compartments are still functional at the time of COCs isolation from the follicular environment. Previous studies reported that approximately 50–80% of the feline COCs carry functionally open GJs [[136], [137], [138]], thus physiologically limiting the number of oocytes that might be positively affected by a pre-IVM treatment. Nevertheless, when gonadotropins are supplemented in the medium, the rate of open GJs does not decrease during the first hours of culture [137], suggesting that a prolonged pre-IVM culture can be conducted. Exploring this possibility will most likely entail the definition of appropriate supplementations and treatments for the meiotic arrest that sustains steady levels of cyclic nucleotides, as analyzed in depth for other mammals.

While in cats the possibility of designing tailored cultural approaches is sustained by several experimental observations and a more or less standardized IVM, in dogs the impossibility of making accurate predictions based on other mammalian models and the lack of standardization in the approach to oocyte culture hinders the development of knowledge based pre-IVM applications.

First, the prolonged in vitro meiotic arrest observed in oocytes retrieved from canine ovaries may recapitulate the in vivo situation of GV-ovulated oocytes, only reaching MII after 2 days of permanence in the oviduct. However, the molecular mechanisms that underlie such atypical regulation of meiosis arrest and resumption have not been elucidated yet.

For instance, one would be tempted to speculate that in vivo ovulated as much as in vitro retrieved dog oocytes have not yet acquired the molecular machinery needed to resume meiosis, somewhat similar to meiotically incompetent gametes such as mice non surrounded nucleus (NSN) or cow GV0. Although this hypothesis might explain, at least in part, the need for several days of continued growth, either in the oviduct or in vitro, to accumulate mRNAs and proteins necessary for re-initiating meiosis, it counteracts the observation that over 90% of ovulated oocytes and 60–70% of those collected from ovaries in the follicular phase show a compact GV chromatin configuration, with discrete condensed foci or one single chromatin clump [120,139,140], which is associated with transcriptional silencing. Therefore, it seems more plausible that these oocytes are subjected to more stringent control of meiotic resumption than other mammals, which may involve additional inhibitory systems within the cumulus cells or the oocyte. That a meiosis-inhibiting secreted factor may be involved is suggested by the observation that canine oocytes isolated during anestrous and cultured in groups of 10 COCs/droplets had higher GVBD and maturation rates than COCs cultured at higher density [141]. The nature and mechanism of action of such an inhibitory system have not been ruled out. Nevertheless, it cannot be excluded that the CNP/NPR2/cGMP pathway is involved, with a CNP secretion sustained by the cumulus cells for several hours.

Another intriguing possibility involves the regulation of translation of stored transcripts coding for proteins involved in meiotic resumption, such as the regulatory subunit of the MPF, cyclin B1 (CCNB1). In the mouse and other animal models, transcripts accumulated during the growing phase are stabilized in complexes with RNA binding proteins (RBPs). When meiosis resumption is triggered, the repressive complex is dissociated, allowing ribosome recruitment and translation [142]. One of the proteins mainly involved in transcripts repression and activation is the CPE binding protein (CPEB), which is responsible for mRNA stabilization at the GV stage, while, upon phosphorylation, it promotes translation of a plethora of transcripts, comprising Ccnb1 [[143], [144], [145]]. While a quota of CCNB1 and the homologous CCNB2 are already expressed in GV mouse oocytes, allowing for a fast meiotic resumption [146], CCNB1 must be de novo synthesized in not murine mammals [147]. In such a scenario, the efficiency of CCNB1 translation and rate of CCNB1 accumulation set the timing of re-entry into meiosis. Indeed, monitoring the expression of CCNB1 in IVM dog oocytes by immunofluorescence shows that it accumulates over 48 h and preferentially in the presence of EGF [148]. However, whether or not mRNA regulation mechanisms are conserved in dog oocytes and their temporal dynamics is unknown. Nevertheless, one possible explanation for the delayed meiotic re-entry may entail the disassembly of complexes responsible for mRNA stabilization and repression.

Regardless of the speculation on the putative mechanisms, it is clear that the prolonged time to meiosis resumption significantly increases the duration of dog oocytes culture, especially if a pre-IVM step is foreseen. Hence particular attention should be paid to the culture environment. Specifically, adding substrates, matrices, or other means to promote a 3D-like environment might be beneficial overall, as suggested in Colombo et al., 2021 [111]. Furthermore, given that

maturation to MII occurs in the oviduct, the IVM medium composition and supplementation should be developed to closely mimic the oviductal fluid in the post-LH days 1–3, while the pre-IVM step should be formulated to recapitulate the follicular hormonal milieu according to the specific folliculogenesis stage, as also implemented for the cow [56,149].

Finally, it should be re-emphasized that also in the dog, COCs collection by ovarian mincing without precise attention to the follicle-of-origin results in a mixed population of gametes that should not be treated as a whole. Indeed oocytes with compact GV chromatin (called either GV III – IV or GV IV – V, depending on the adopted classification) are preferentially retrieved upon ovulation or from ovaries at the follicular stage [139,140] and from larger follicles [150]. These gametes will likely not need or not benefit from a pre-IVM culture. Conversely, COCs retrieved from anestrous ovaries and smaller follicles are more likely to be at earlier stages of development, given the looser chromatin structure and smaller diameter [140,150]. A pre-IVM or a prolonged culture might be necessary in this case to allow growth and final differentiation.

In the absence of non-invasive markers that allow clustering of oocytes with different potential, we can only recommend stratifying the gametes following this guiding principle: type of ovary (follicular, luteal, anestrous phase) > diameter of the follicle > diameter of the oocyte, to obtain sub-populations enriched in the compact or loose GV chromatin configuration and destine them to tailored culture systems.

However, it should be noted that the estrous cycle influences the GJ coupling significantly, with COCs collected from anestrous ovaries showing functionally closed GJ [123,124]. Given the fundamental role of junctional coupling for oocyte growth and differentiation and that most of the ovaries obtained from ovariohysterectomy and destined for research are anestrous, this might represent a significant limitation to the development of pre-IVM treatment in dogs.

Concluding remarks

The bovine model has been highly informative and inspiring in the past four decades to translate reproductive technologies into other species. Most of the recent advances in ART have come from studies in cattle, particularly those revealing the importance of several processes that must be recapitulated in vitro to ensure the proper development of the oocyte. In cattle, hormonal synchronization [[151], [152], [153]] and a tailored in vitro culture system [74] could lead to an increase in developmental competence and reproductive efficiency.

The prematuration approach has become a tool that today is part of the repertoire for treating human infertility, particularly in those patients that can significantly benefit from IVM treatments such as ovarian hyperstimulation syndrome (OHSS) and polycystic ovarian syndrome (PCOS) [154]. Recently, prematuration became effective in clinical approaches with the so-called capacitation step (CAPA-IVM) [155]. The CAPA-IVM improved maturation success and produced better-quality embryos, and oocytes from follicles <6 mm benefitted the most from pre-IVM treatment [156], resulting in live birth following embryo transfer [157].

Deepening the basic knowledge and comparative aspects of the oocyte's biology is paramount since they bring on the way of translational application, as recently outlined in the paradigmatic odds and similarities between human and bovine oocytes and embryos [158,159], the only way to get successfully translated between species.

In conclusion, in the face of the limited number of studies, advancement of knowledge on the carnivore's oocyte biology, particularly the canids, could lay the bases of more tailored approaches given the physiological characteristics of these species. It would be desirable to build a unifying framework of oocyte biology to boost fertility performance in the breeding system, biodiversity preservation, and translational medicine.

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Declaration of competing interest
The authors have no conflicts of interest to disclose.

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