Theriogenology 158 (2020) 105–111

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Embryonic diapause in roe deer: A model to unravel embryo-maternal communication during pre-implantation development in wildlife and livestock species

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ARTICLE INFO

Article history:
Received 23 June 2020
Accepted 27 June 2020
Available online 6 July 2020

Keywords:
European roe deer (*Capreolus capreolus*)
Embryo-maternal communication
Pluripotent stem cells
Species conservation
Embryonic diapause

ABSTRACT

An alarming number of large mammalian species with low reproduction rates is threatened with extinction. As basic knowledge of reproductive physiology is currently lacking in many species, increasing the understanding of reproductive physiology is imperative and includes the development of novel artificial reproduction technologies. Despite the relatively comprehensive knowledge on molecular mechanisms underlying reproduction in livestock species such as cattle, pregnancy failures are likewise far from understood. Contrary to other wildlife species, the European roe deer (*Capreolus capreolus*) displays a remarkably high pregnancy rate. In parts, cattle and roe deer exhibit comparable features of preimplantation embryo development. Therefore, understanding the high fertility rate in the roe deer holds a great potential for cross-species knowledge gain. As the only known species among the artiodactylae, the roe deer displays a long period of embryonic diapause. The preimplantation blastocyst reaches a diameter of 1 mm only at around 4 months compared to around 13 days post estrus in cattle. The expanded blastocyst survives in a uterine microenvironment that contains a unique set of yet unidentified factors that allow embryonic stem cells to proliferate at low pace without impairing their developmental potential. Upon reactivation, intimate embryo-maternal communication comparable to those reported in cattle is thought to occur. In this review, current knowledge, parallels and differences of reproductive physiology in cattle and roe deer are reviewed. The roe deer is proposed as a unique model species to (1) enhance our knowledge of fertility processes, (2) define factors that support embryo survival for an extended period, (3) advance knowledge on embryonic stem cells, and (4) unravel potential implications for the development of novel strategies for artificial reproductive technologies.

1. Introduction

Fertility problems in livestock and wildlife most often lead to a premature production dropout and extinction, respectively [1]. In heifers, beef cattle, moderate yielding dairy cows, roe deer does and camelids, fertilization rates are generally high, reaching up to more than 90% [1]. Yet, a large proportion of embryonic losses occur post fertilization [2]. Approximately 8% of pregnancies are lost after implantation, specifically between days 30 and 90 of gestation [2]. Embryo and fetal mortality rates lie between 40 and 56% in heifers and high-producing dairy cows, respectively [1]. This shows that most pregnancies are lost prior to implantation. A failure of embryo-maternal communication and maternal recognition of pregnancy (MRP) has been widely accepted as underlying cause [1,3,4]. Despite intensive research aiming at improving pregnancy rates, there is currently no prospect of its improvement.

In wildlife, the rate of population decline in larger species calls for action [5–7]. Breeding programs of nearly extinct species have been introduced [8,9], and research has largely focused on employing new artificial reproductive technology (ART) tools, to preserve the biodiversity, and multiply the genetics once available [6,7,10–12]. The number of animals within a population varies as a result of various factors, including predator to pray and host to parasite interactions, but also conflicts with humans or wars affecting geographic range and habitat [6,13,14]. A low reproduction rate increases the risk of extinction for animals with a body weight of more than 5.5 kg [15]. In many wildlife species, little

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https://doi.org/10.1016/j.theriogenology.2020.06.042

detail is known about reproductive physiology. Contrary to other wild animals with a body weight of more than 5.5 kg, the European roe deer (*Capreolus capreolus*) displays remarkably high pregnancy rates. The average number of implanted fetuses and live-born fawns in both captive and wild population lies between 1.4 and 2.0 per female [16–18]. The offspring female:male sex ratio has been reported as 1:1 [19], and twins were found to be mostly non-homozygous [20]. The roe deer is the only known ungulate that displays embryonic diapause in form of a delayed implantation. In Bischoff's large observational study dating back to 1854, the roe deer buck was documented to provide functional semen in June, July and August [21]. Rut, copulation and fertilization were reported to take place between the end of July and end of August [21]. A fertilized egg was found in the oviduct until mid-August and persisted free floating in the uterus as expanded blastocyst until the end of December [21]. After this prolonged period of diapause, embryonic development in roe deer was found to resemble that of other ruminants [21,22], namely trophoblast elongation, apposition, attachment, implantation and synepitheliochorial placentation. Since then, numerous reports have confirmed these initial findings.

The need for adequate embryo-maternal interactions during the establishment of a pregnancy emphasizes the urge for a better understanding of pregnancy losses. Understanding early embryo-maternal communication is a difficult task, as one cannot separate one communication partner from the other without disturbing their interaction. The set of signaling factors used by the embryos and maternal tissues are in part overlapping, adding an additional layer of complexity to understanding the signals' origin and respective responses. Lately, numerous studies have emphasized on understanding this interaction by investigating embryonic and endometrial transcriptome changes and by characterizing the uterine microenvironment in several species. Nevertheless, existing gaps in knowledge must be closed to explain the high proportion of early pregnancy losses and reduced fertility in wildlife species. In this review, we will specifically focus on reproductive characteristics of both cattle and roe deer. We aim to delineate why research in the roe deer as an exemplary wildlife species can increase our understanding of general aspects of fertility, including embryo-maternal communication during pre-implantation, and to provide new insights into potential novel ART strategies.

2. Pregnancy establishment in cattle and in European roe deer comprising embryonic diapause

2.1. The estrous cycle

The estrous cycle of mammalian species consists of a follicular (proliferative) and a luteal (secretory) phase. In cattle, there are two to three follicular waves, which are preceded by an increase in follicle stimulating hormone (FSH) [23]. Stimulated by estrogens from growing follicles, a luteinizing hormone (LH) surge induces ovulation, which results in the formation of a corpus luteum (CL) and the rise of peripheral progesterone (P4) from day 2 post estrus onwards [23]. The CL provides sufficient peripheral progesterone (P4) throughout the luteal phase to maintain pregnancy [23]. In cyclic cattle, the follicular phase is much shorter than the luteal phase, namely 4–5 and 14–18 days, respectively. Around day 17–18 post estrus, endometrial prostaglandins induce luteolysis and the peripheral P4 concentration drops rapidly to induce another cycle [23]. During pregnancy, luteal P4 production remains high and only a minor placental P4 contribution has been reported [24]. Unlike in cattle, the roe deer is a mono-estrous species and does not display luteolysis during the period of embryonic diapause or subsequent post-implantation gestation [20,25]. Ovulation has been shown to be seasonal and under the control of melatonin and an average of 2.13 ovulations per doe have previously been reported [26,27]. Like in cattle, an LH surge precedes ovulation and the CL secretes P4 [20,28,29]. The number of CL has been shown to neither correlate with peripheral, nor uterine tissue P4 concentration [30]. During the pre-implantation period, plasma and uterine tissue P4 remained stable [30,31], while the uterine progesterone receptor (PR) expression decreased at the beginning of December upon prolonged progesterone exposure [32]. After implantation, progesterone increased, suggesting at least a partial contribution of the developing placenta [33–37].

2.2. Embryo development

Across mammalian species, key events of early embryo development are conserved. These include the fertilization of the oocyte and embryonic genome activation (EGA) in the oviduct. In many species, the embryo travels to the uterus, where the formation of a blastocyst takes place, while in other species like camelids, the embryo forms a blastocyst in the oviduct. Upon expansion, the blastocyst hatches from the zona pellucida. Specific to ungulates, the trophoblast elongates prior to implantation. It thereby forms a long tubular structure which fills the entire uterine horn. The resulting large contact area with the endometrium facilitates the anti-luteolytic signal transmission necessary to prolong luteal support.

The bovine embryo reaches the uterus at the morula stage between day 4 and 6 after ovulation [38]. Here, the embryo forms a blastocyst and hatches at day 8, becomes ovoid between day 12 and 14, and elongates until day 16 [38,39]. During the pre-implantation embryo development, the embryo not only undergoes morphological, but also numerous transcriptional changes [40–49]. The variation in embryonic size at a specific day of development increases with developmental progression [50]. It is not until day 13 that the embryo reaches a diameter of 1 mm [50]. At the onset of elongation, the embryonic size greatly varies from several millimeters up to several centimeters [50]. Up to the blastocyst stage, the embryo develops relatively autonomously [51,52], and it has been shown elegantly that uterine secretions are necessary for embryo elongation [53]. On day 18 of pregnancy, the bovine embryo starts to implant and the direct exchange of nutrients, oxygen and metabolites with the mother takes place following placentation.

In roe deer, fertilization takes place in July/August, while embryo elongation and implantation occur only 5 months later in December/January [20]. This indicates the obligate period of embryonic diapause in this species [20]. Our own research in roe deer focusing on the period of diapause is currently based on the study of more than 500 does (own unpublished data, Table 1). For this cohort sampled between 2015 and 2018, we estimated the distribution of embryonic growth as displayed in Fig. 1 [54]. Unlike bovine embryos, the majority of roe deer embryos reached the size of 1 mm only within the first half of November, corresponding to roughly 4 months after fertilization (Fig. 1) [54]. While the first implantation in our cohort was observed in the period between 1 and 14th of November, the majority of embryos had implanted by the beginning of January (Fig. 1B) [54]. This demonstrates the largely prolonged pre-implantation period in roe deer compared to cattle. Notably, while the cohort of embryos had a rather uniform size until the first half of October, the size distribution increased in the further course of time. Likewise, the onset of elongation varied largely between the first half of December until mid of January (Fig. 1A). Thus, the roe deer offers a unique model to study embryo-maternal communication during pre-implantation with a high time resolution. We hypothesize to identify important factors for

Table 1
Cohort statistics of all sampled adult roe deer does.

Parameter	Value	Min	Max
Sampling periods 2015-2018		Sept 1	January 15
Sampled roe deer does	546 [#]		
Does with pre-implantation embryos	487 [#]		
Does with implanted embryos	59 [#]		
CL per doe	1.96 ± 0.02 [mean ± SEM]	1 [#]	4 [#]
Embryos per CL	0.78 ± 0.02 [mean ± SEM]	0 [#]	1 [#]
Pre-implantation embryos per doe	1.52 ± 0.02 [mean ± SEM]	0 [#]	4 [#]
Implantated embryos per doe	1.83 ± 0.06 [mean ± SEM]	1 [#]	3 [#]
Elongated embryo recovery	90 [%]		
Pregnancy rate	92 [%]		

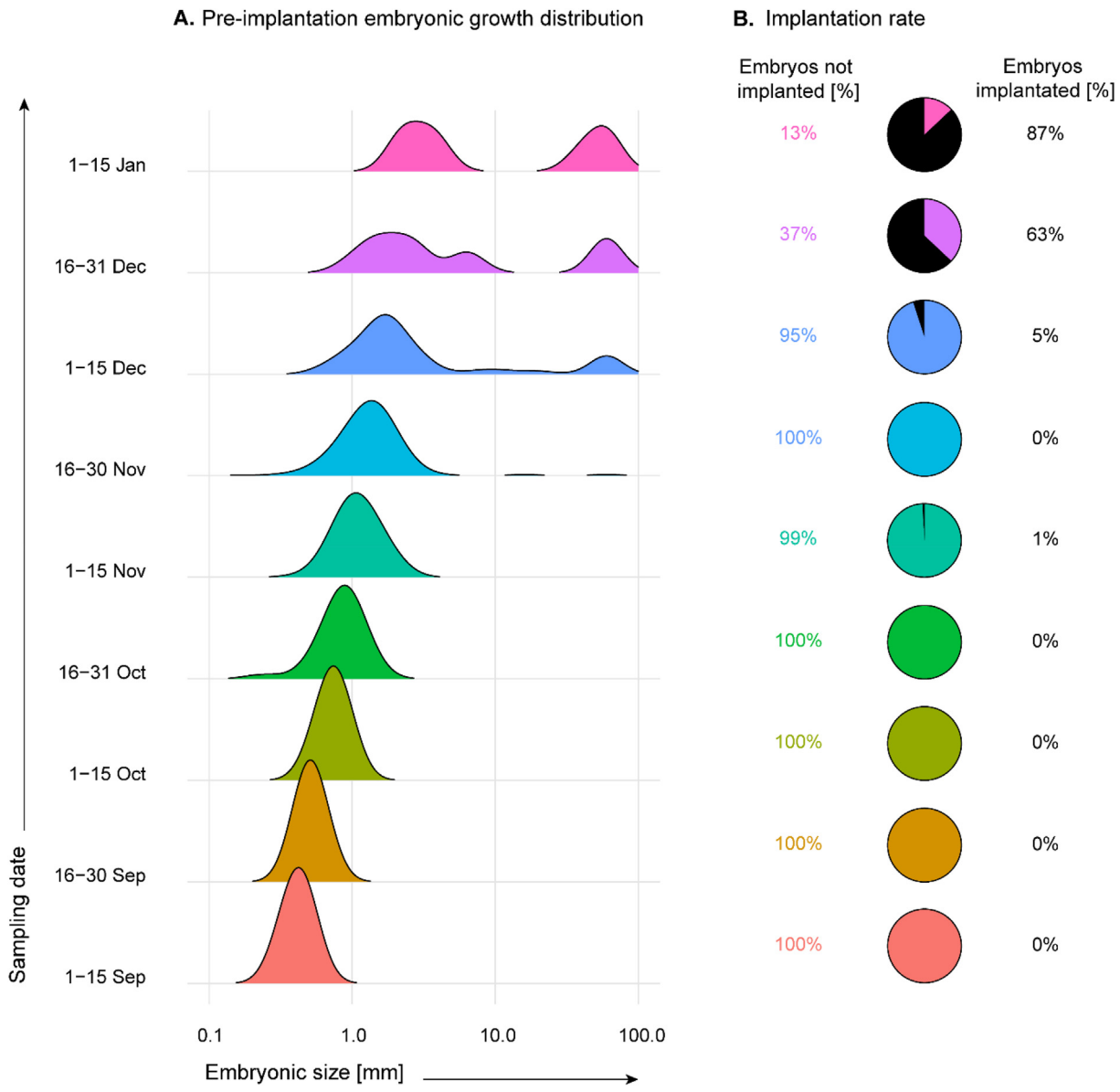

Fig. 1. Embryonic growth during diapause and upon resumption of embryo development in the Euproean roe deer [54]. **A.** The growth distribution curves (own unpublished data from field sampling of 546 does of at least one year of age between 2015 and 2018) are displayed for each sampling date interval. To allow plotting of the embryonic growth distrubtions for each sampling date interval, the area of the embryonic size distribution was normalized to 1, assuming equal sample numbers for each interval. The density plots were plotted with geom_density_ridges in R version 3.6.1. Geom_density_ridges is embedded in ggplot 2 [55], which arranges multiple density plots in a staggered fashion. **B.** (Pre)-implantation rates for each date interval are indicated in a pie chart and as percentage. The color-matched percentage on the left-hand side of the pie chart for each sampling date interval indicates the percentage of pre-implantation embryos, while the percentage on the right-hand side of the pie chart in black indicates the percentage of implanted embryos which are not displayed in Fig. 1 A. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

embryo elongation and potentially those involved in the establishment of pregnancy.

From our cohort, the sample statistics are provided in Table 1 (own unpublished data). The embryo recovery rate calculated as recovered embryos per CL was 0.78 and each pregnant doe carried on average 1.5 embryos. The recovery of the elongated embryos specifically was 90%, while the implantation rate was as high as 92%. This indicates that although some embryos were not recovered by flushing, the pregnancy rate was as high as 92%.

It has previously been reported that the post-implantation development lasts another four to five months, and an average of two fawns (range 1–3) are born in May [20].

2.3. Embryo-maternal interactions

Adequate developmental-stage specific interactions with the endometrium are required to allow successful implantation [56]. The development of embryos past the stage of the hatched blastocyst stage has so far proven impossible to achieve *in vitro* [51–53]. It is hypothesized that yet unidentified maternal signals are responsible for further development failures. Around the time of elongation, species-specific physical and chemical properties of the embryo enable appropriate MRP. The latter constitutes of either an anti-luteolytic or a luteotropic mechanism, both ensuring pregnancy maintenance. From the time of hatching, the embryo releases large amounts of interferon τ (IFNτ), reaching strinkingly high quantities with elongation and a maximum around day 18 [4,56]. In the roe deer, luteal oxytocin remains stable and does thereby not secrete luteolytic prostaglandin F2α of endometrial origin reaching the CL [25]. In accordance with this, and potentially related to the mono-estrous behavious, neither an anti-luteolytic signal, nor a luteotropic signal has been described to date. As only ruminant species known to date, IFNτ is absent in roe deer and pregnancy is maintained despite its absence [32,57]. However, roe deer embryos have been shown to secrete roe deer-specific pregnancy associated glycoproteins (PAGs) upon implantation as detected in the maternal blood [36]. As most endometrial transcriptome changes in bovine around the period of MRP are IFNτ-induced, the roe deer as model organism may thus allow the identification of conserved non-IFNτ-related embryo-maternal communication signals.

2.4. Embryonic transcriptome changes

Highly dynamic and developmental stage-specific transcriptional changes have been observed in pre-implantation embryos [40,44,45,47,58,59]. Key steps include EGA and embryo elongation. Graf et al. (2014) examined transcriptome changes during bovine embryo development [40]. To facilitate parent-specific transcriptome analysis and thereby allow for identifying when EGA takes place, they used *in vitro* fertilization of *Bos taurus taurus* oocytes with sperm from a *Bos taurus indicus* bull [40]. For the transcriptome analyses, they used germinal vesicle and metaphase II oocytes, and embryos at the four-cell, eight-cell, 16-cell, and blastocyst stages, and found that EGA occurs at the 8-cell stage [40]. At the time of EGA, single cell transcriptome analysis showed an asynchronous development of single blastomeres [47]. Thus, embryo development is highly dynamic as such, and at a given developmental stage, single embryonic cells display different developmental progressions. The initiation of embryo elongation in cattle is characterized by the expression of genes involved in the 'interactions with the extracellular matrix' and genes of the 'matrix metallopeptidase family', which is indicative of preparation for embryo implantation [44]. In addition to transcriptome changes during key steps, continues dynamic changes have been shown by a study that investigated the embryonic transcriptome of day 7, 10, 13, 16 and 19 bovine embryos [59].

Up to date, pre-implantation roe deer embryonic gene expression changes have not been reported. In line with the increased transcription of genes involved in proliferation and protein synthesis, it has been shown that the mitotic rate in diapausing roe embryos was low, yet increased with embryo size [20–22,60]. Bromodeoxyuridine (BrdU) incorporation was less than 5% during diapause, whereas it increased to 10–15% at later stages of diapause [60]. The *de novo* protein synthesis was 22.5–32.7-fold higher in elongated compared to diapausing embryos [27,35]. This indicates a strong activation of developmental pace between the diapause and elongated stage. Embryo development has been shown to be dynamic in various ungulates, and changes are particularly evident between the hatched blastocyst and elongated embryos. We hypothesize a transcriptional dynamic process during the pre-implantation embryo development in roe deer, while most changes are coinciding with embryo elongation.

2.5. Endometrial transcriptome changes

Embryo-maternal communication and the perception of embryonic signals at MRP results in numerous DEG in the bovine endometrium [4,61–63]. More recently, these changes have been shown to be cell-type-specific [64,65]. On day 18 of bovine pregnant compared to cyclic heifers, 109 DEG were higher in the endometrium of pregnant animals, whereas 70 DEG were higher in cyclic animals [61]. A total of 41 DEG that were higher in the pregnant versus cyclic animals, were previously found to be induced by interferons [61]. The other pregnancy-induced DEG were involved in 'regulation of transcription', 'cell adhesion', 'modulation of the maternal immune system' and 'endometrial remodelling' [61]. Cells-type-specific endometrial transcriptional changes have been observed to largely coincide with embryo elongation [4,61,63–66]. In cattle, embryonic IFNτ induces the expression of both classical and non-classical interferon-induced genes [4]. Contrary to the increased expression of interferon-induced genes in cattle, we evidenced a lower abundance of the classical IFN-stimulated genes *IRF2*, *MX1* and *ISG15* in the presence of an elongated roe deer embryo [32]. In addition, we reported that roe deer endometrial cell types respond differently to the presence of an elongated embryo [32]. We showed that the LE displayed developmental stage-specific clustering and a uterine loss of the PR was apparent after the beginning of December [32]. Thus, we proposed that the uterine loss of PR potentially plays an important role in embryo elongation, the receptivity of the endometrium, as well as preparation for implantation [32].

2.6. Uterine microenvironment

The uterine microenvironment, comprising the uterine fluid, constantly changes during early embryo development to support its survival and the establishment of pregnancy. The uterine fluid presents a mixture of signals from embryonic and endometrial origin, and it contains proteins, amino acids, nutrients, ions and metabolites [67–69]. Proteins in the uterine fluid are essential for embryo development past the blastocyst stage and are hypothesized to support embryo elongation [70]. Uterine amino acids [71] sustain embryo development and survival by supplying energy [72], facilitating protein and nucleotide synthesis [73], regulating the pH and osmolarity [74,75], as well as by their antioxidant capacity [76]. The bovine embryo was found to display an increased pyruvate and glucose uptake and increased lactate production during developmental progression from the 2-cell stage to the hatched blastocyst stage [77]. With developmental progression, the

bovine embryo has been shown to consume increasing amounts of aspartate, glutamate, serine, threonine, arginine, methionine, isoleucine and leucine, whereas concomitantly producing increasing amounts of glutamine, glycine, alanine, tyrosine, tryptophan and phenylalanine [77]. In the bovine uterine fluid during pregnancy, the most abundant amino acids were threonine and glycine [78,79].

In roe deer, the uterine secretions were up to 1.5-fold increased during implantation compared to diapause [35,80], and a rise in uterine fluid hexose, fructose, total protein, α-amino nitrogen and calcium coincided with embryo elongation [33,81,82]. Recently, we have identified and quantified 819 proteins in the uterine fluid [83]. In line with the importance of uterine proteins in embryo elongation [84], most differentially abundant proteins were identified in the uterine fluid between early diapause and elongation [83]. The proteins with a significantly lower abundance at elongation than during diapause were involved in cellular detoxification, while proteins with a higher abundance at elongation were indicative of a support of proliferation [83].

2.7. Pluripotent stem cells

The embryonic microenvironment plays an important role in keeping cells in a pluripotent state. It has previously been shown that mouse embryonic stem cells can be maintained as naïve pluripotent cells in the presence of MEK and GSK3 inhibitors [85]. The epiblast of diapausing mouse embryos has been shown to maintain all features of naïve pluripotency [86]. The regulation of embryonic diapause in mice is initiated hormonally, evidencing that maternal endo-/paracrine changes can affect the embryonic developmental pace. In addition, MYC-depleted stem cells have been shown to enter dormancy and MYC-depleted embryos enter embryonic diapause [85]. Likewise, the inhibition of mechanistic target of rapamycin (mTOR) induced a reversible state of embryonic diapause in mouse embryos [87]. The latter embryos were shown to remain pluripotent and were able to give rise to embryonic stem cells [87]. This highlights that MYC and mTOR, via factors in the embryonic microenvironment, affect embryonic developmental pace.

Mammalian species that display diapause show a distinct presence of factors implicated in the regulation of diapause. While endocrine uterine stimulations causing the resumption of embryo development have been shown to be species-specific, embryonic molecular factors seem conserved [88]. Up to date, the key factors involved in the regulation of embryonic diapause in roe deer have not been described. Especially the low developmental pace of the roe deer embryo offers the opportunity to obtain pluripotent stem cells and to study stem cell dynamics. Moreover, the roe deer embryonic microenvironment is highly interesting, as roe deer embryos display physiologically reduced cell proliferation – free of damage – over a long period of time in a medium of yet unknown composition.

3. Artificial reproductive technologies

In cattle, pregnancy success rates are 34% for *in vitro* produced versus 42% for *in vivo* embryos, which were transferred between day 6 and 7 after fertilization [89]. This might, in parts, be explained by embryonic gene expression of for example the apoptosis gene *BAX*, IFNτ and E-cadherin [90,91], and metabolic differences between *in vitro* produced and *in vivo* embryos [44,90]. Especially for endangered species, it is challenging to have the female and male gametes at disposal at the same place and time [92]. A recent review by Comizzoli and Holt (2019) highlighted the research areas, technologies and approaches that will significantly contribute to species conservation [10]. Examples include the use of ART to save endangered species like the Rhinoceros [93], the long-term storage of oocytes and spermatozoa at room temperature [94,95], and germ cell transplantation [96]. By identifying the uterine fluid composition in the roe deer that facilitates embryo survival for a prolonged period of time, we may contribute to the development of novel "halting" media. These media could be used as promising alternative preservation options for oocytes, spermatozoa and embryos. In addition, technical challenges related to for example stem cells could be addressed in large non-endangered species [97].

4. Outlook

Future comparative transcriptomics studies are envisaged to shed light on the biological background of early embryonic losses in cattle. Understanding the high fertility rate in roe deer thereby holds a great potential for knowledge gain. Defining the embryonic microenvironment, e.g., proteins [83], amino acids and metabolites, that support embryo survival, but highly decelerates it, could potentially contribute to novel strategies for ART in wildlife and livestock. Research should not be limited to defining optimal culture conditions, but should further emphasize on embryonic stem cells. Here, diapausing embryos are of specific interest, as mouse diapausing epiblasts have previously been shown to maintain all features of naïve pluripotency [86]. Thus, diapausing embryos offer an invaluable tool for obtaining pluripotent stem cells of a wide variety of species.

In conclusion, the roe deer as a large animal model offers the opportunity to increase our understanding as to why early embryonic development is so vulnerable, how novel culture conditions can be defined to prevent preservation-induced embryo damage, and to advance research in the field of pluripotent stem cells.

Funding

This study was funded by the Swiss National Science Foundation SNSF (310030_185026).

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

We kindly thank the local hunters for generously providing us with sample material and all colleagues who participated in huntings for sample collection. The authors are active participants of the COST Action CA16119 (CellFit - In vitro 3D total cell guidance and fitness).

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