

## Introduction to Pregnancy in Waiting: Embryonic Diapause in Mammals

### Proceedings of the Third International Symposium on Embryonic Diapause

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The capacity of the mammalian embryo to arrest development during early gestation is a topic that has fascinated biologists for over 150 years. The first known observation of this phenomenon was in a ruminant, the roe deer (*Capreolus capreolus*) in 1854, later confirmed in a number of studies in the last century [1]. The phenomenon, now known as embryonic diapause, was then found to be present in a wide range of species and across multiple taxa. Since that time, its biological mystery has attracted studies by scientists from around the globe.

The First International Symposium on the topic of embryonic diapause in mammals was held in 1963 at Rice University, Houston, Texas. It resulted in a proceedings volume entitled “Delayed Implantation”, edited by A.C. Enders [2]. The symposium was distinguished by the novel recognition of that era that a wide range of species had been identified with embryonic diapause, including rodents, marsupials and carnivores. The emerging technology of the time, particularly structural approaches, permitted new understanding of the events of diapause and embryo reactivation. The newest methods provided key data on the temporal window of implantation in rodents, introduced new physiological approaches, and illustrated some of the first transmission electron microscope investigations of the blastocyst.

The Second International Symposium on Embryonic Diapause was held in Thredbo, Australia in 1980. The proceedings entitled “Embryonic Diapause in Mammals”, edited by A.P.F. Flint, B.J. Weir and M.B. Renfree [3] were published in 1981 as Supplement 29 of the Journal of Reproduction and Fertility. As with the previous meeting, the diversity of species displaying diapause was highlighted. The research contributions specifically exploited maturing technology to study reproductive processes, particularly the capacity to accurately measure hormones and the advances in imaging. Among the highlights of that meeting were studies that reported the essential role of prolactin as a negative regulator on the one hand, maintaining diapause in marsupials [4] and, on the other, as a positive activator, terminating diapause in a carnivore species [5].

Some 39 years had passed since the last symposium on the topic of diapause, and the impetus for convening a new congress on the subject came from a meeting between the current organizers in 2017. After some discussion and a number of Skype conferences across three continents, it was agreed that the meeting would be organized in Switzerland at ETH Zurich. A joint application for funding to hold the Symposium at Congressi Stefano Franscini,

Monte Verità, Ascona Switzerland was successful, and funding was acquired from ETH Zurich, the Swiss National Science Foundation and additionally, from the Society for Reproduction and Fertility. Again, the Symposium was truly interdisciplinary as well as international, in that the delegates hailed from four continents and from universities, research institutes, zoos and industrial settings. We judged it important to acknowledge the major contributions over the last 50 years of three pioneers in research on embryonic diapause, namely, Drs. Allen Enders, Rodney Mead and S.K. Dey (Figure 1). We therefore invited each to provide a short video recording. Their research insights into the enigma of diapause provided a wonderful introduction to the meeting. Their videos can be found at the following link: <https://www.diapause2019.ethz.ch/abstract-submission/>.



**Figure 1. Pioneers in the study of embryonic diapause.** Left to right: Allen C. Enders, Rodney A. Mead and Sudhansu K. Dey.

Much has changed in research on the topic of embryonic diapause since 1980. A wealth of new information has become available from laboratories all over the globe, in a number of unrelated species and from multiple perspectives. For this reason, the overall goal of the Third Symposium was to provide a 21st century view of the events and regulation of diapause. The meeting successfully brought together scientists from multiple disciplines and with a variety of interests, including bench and field biologists, exotic species specialists, cell biologists and stem cell researchers (Figure 2, see Appendix A for contact information). It further served to highlight the new molecular techniques now available. Among the specific foci of the Symposium was to explore new findings on the structural and molecular changes that take place in the embryo upon entry into its arrested state. It was also of interest to examine how the embryo is maintained with little or no developmental progression during diapause, as well as the mechanisms of and sequelae to its reactivation. As the uterine environment represents the proximal control of diapause, a further specific goal was to acquire an up-to-date panorama of uterine regulatory mechanisms that promote developmental arrest and subsequent reactivation of the embryo. We now know that there are remarkable commonalities in the mechanisms of control of diapause among the three best studied species, the mouse, the mink and the tammar wallaby [6-8]. Thus, it was our intention that, by close examination of a diverse range of species with diapause, the symposium could shed light on the evolution of the trait of diapause, whether it evolved once, or whether its presence in unrelated taxa demonstrates parallel or convergent evolution. Finally, as the first embryonic stem cells were derived from the inner cell mass of rodent embryos in diapause, gaining insights into stem cell pluripotency and differentiation was a significant goal of the Symposium.

A further important aim of the symposium was to pass the baton to the new generation of researchers studying diapause. In this context, three young investigators were invited as the keynote speakers, representing state-of-the-art research in rodent, marsupial and carnivore diapause. As with previous symposia, species diversity was an important issue, with presentations on topics that ranged from seals to wolverines and bats. The presence and characterization of diapause in endangered species, particularly the giant panda, were also topics of presentation and discussion. To unravel the mysteries of diapause, the traditional approaches of structural biology, cell culture, hormonal quantification and imaging technology were complemented by state-of-the-art molecular methods, ranging from global transcriptome and proteome analysis to *in situ* hybridization and in-depth lipid and carbohydrate analyses.

The Congressi Stefano Franscini sponsored two prizes for the best presentations by a young scientist. Michelle Shero received the presentation prize for her study on seals. The best poster prize was shared by Vera van der Weijden for her work in roe deer and Lukasz Gasior for his poster on transcriptomics of mouse embryos in diapause.

From the 35 abstracts of oral and poster contributions to the symposium (Appendix B), some 15 manuscripts were submitted for inclusion in this volume of the proceedings. Some who presented preliminary and primary data chose not to submit, and some presentations on the same topic or species were consolidated into a single manuscript. All manuscripts were subject to peer review for content, quality and originality. The edited versions of the discussion of the presentations, where manuscripts were submitted follow each article.



**Figure 2. Attendees at the III International Symposium on Embryonic Diapause.** From left to right: Michelle Shero, JeeYeon Cha, Jessye Wojtusik, Anna Rüegg, Erin Curry, Jane Fenelon, Helen Bateman-Jackson, Chris Murphy, Arnab Banerjee, Simona Bisogno, Aydan Karslioglu, John Rasweiler, Roberta Arena, Jella Wauters, Toshihiko Fujimori, Grazyna Ptak, Kirsten Wilson, Susanne Ulbrich, Hans-Werner Denker, Lukasz Gasior, Katarina Jewgenow, Anna Hankele, Joanna Rudnicka, Szymon Gawel, Marilyn Renfree, Federica Zacchini, Bruce Murphy, Vera van der Weijden, Kinga Fic, An Junhui, Sergio Ruiz Macias, Yisi Hu, Cai Kailai, Barbara Drews, Stephen Frankenberg. Missing from the photo: Colin Stewart, Pierre Comizzoli, Thomas Hildebrandt, Dorota Niedzwiecka.

We, the Organizing Committee of the III International Symposium on Embryonic Diapause (Figure 3), express our sincere thanks to the participants and the sponsors of the Symposium, all of whom contributed to its resounding success. We are grateful to the many reviewers who evaluated the manuscripts, and Jim Pru for his aid with videoing. Special

thanks go to Dorota Niedzwiecka for her extensive efforts in assembling these proceedings for publication.



**Figure 3.** The Organizing Committee of the III International Symposium on Embryonic Diapause. Left to right, Susanne Ulbrich, Katarina Jewgenow, Marilyn Renfree and Bruce Murphy.

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## Appendix B. Abstracts of presentations at the symposium

### Platform presentations

#### A role for *Msx* genes in mammalian embryonic diapause

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Mammalian embryonic diapause is a phenomenon defined by the temporary arrest in blastocyst growth and metabolic activity within the uterus which synchronously becomes quiescent to blastocyst activation and implantation. This reproductive strategy temporally uncouples conception from parturition until environmental or maternal conditions are favourable for the survival of the mother and newborn. The underlying molecular mechanism by which the uterus and embryo temporarily achieve quiescence, maintain blastocyst survival and then resume blastocyst activation with subsequent implantation remains unknown. We found that uterine expression of *Msx1* or *Msx2*, members of an ancient, highly conserved homeobox gene family, persists in three unrelated mammalian species during diapause, followed by rapid downregulation with blastocyst activation and implantation. Mice with uterine inactivation of *Msx1* and *Msx2* fail to achieve diapause and reactivation. Remarkably, the North American mink and Australian tammar wallaby share similar expression patterns of MSX1 or MSX2 as in mice - it persists during diapause and is rapidly downregulated upon implantation.

To understand the cause of delayed implantation in mice, we compared proteome profiles between floxed and *Msx*-deleted uteri. In deleted uteri, several functional networks, including transcription/translation, ubiquitin-proteasome, inflammation, and endoplasmic reticulum stress, were dysregulated. Computational modeling predicted intersection of these pathways on an enhanced inflammatory signature. Further studies showed that this signature was reflected in increased phosphorylated I $\kappa$ B levels and nuclear NF $\kappa$ B in deleted uteri. This was associated with enhanced proteasome activity and endoplasmic reticulum stress. Interestingly, treatment with anti-inflammatory glucocorticoid (dexamethasone) reduced the inflammatory signature with improvement of the diapause phenotype.

These studies provide strong evidence that the *Msx* gene family constitutes a common conserved molecular mediator in the uterus during embryonic diapause to improve female reproductive fitness by limiting aberrant inflammatory responses.

## Embryonic diapause: a developmental breather

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Embryonic diapause in mammals is a temporary delay or halt in early embryo development. It is remarkable for its precise control of embryonic cell division and metabolism, but the underlying molecular control mechanisms are still being discovered. Diapause has been reported in about 130 species of mammals from a wide range of groups, ranging including some rodents, carnivores, deer, bats, moles and 38 marsupial species but only one ungulate. From an evolutionary perspective, diapause provides a mechanism to regulate the time of birth or independence of the young to maximise reproductive success. Physiologically, it provides a mechanism to synchronise early embryo development with uterine changes. The tammar wallaby is a master of embryonic diapause, during which the blastocyst normally lies dormant, with low metabolism and no cell division or cell loss, for 11 months until a seasonal signal transduced by a neuroendocrine pathway reliably reactivates the uterus and blastocyst. Recent studies are clarifying some of the molecular dialogue between the uterus and blastocyst during diapause and reactivation, including LIF, PAF, MSX, FOXO, EGF and HDGF, factors implicated in the control of diapause in other mammalian species and highlights new avenues for understanding this fascinating phenomenon.

## Molecular cues of diapause regulation in the European roe deer (*Capreolus capreolus*)

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More than 150 years ago, the European roe deer (*Capreolus capreolus*) was the first species in which Ziegler 1843 and Bischof 1854 described the phenomenon of embryonic diapause. Thereby, the puzzling discrepancy between the breeding season of roe deer in July/August and the first appearance of the fetus in the uterus not earlier than January was resolved. Principally, it was assumed that the most advantageous time for mating is when animals are in an optimal nutritional state. The prolonged pregnancy then allows birth to take place when the offspring have the best chance of survival. To date, diapause is known to occur in a wide range of species from different mammalian orders as a temporary delay or halt of embryonic development. To that end, diapause highlights striking similarity to cellular dormancy as quiescent state of non-proliferating cells reacting to a specific environmental condition. However, it is unclear why some species have adopted a diapause, while closely related ones did not. It is exciting to presume conserved molecular mechanisms regulating diapause across species, next to species-specific characteristics. During preimplantation development, the embryo is nourished by the intrauterine environment, namely the histotroph, comprising of maternal and embryonic secretions. The histotroph plays a crucial role in providing a milieu permissive to the needs of the embryo and thereby may decisively govern the developmental velocity of the embryo. It is here that embryonic and maternal secretions compile and signals may be intercepted. The roe deer as only ungulate known to display diapause represents an interesting model for research in bovine, as the preimplantation development, embryo elongation and the epitheliochorial cotyledonarian placentation are shared between the two closely related ruminant species. However, while diapause is not present in bovine, in roe deer the pace of pre-implantation development is largely reduced. Remarkably limited knowledge is available to date and neither the signals regulating entry into roe deer diapause nor the factors responsible for embryonic reactivation are known. Our lab recently started large-scale *ex vivo* field studies of the roe deer to unravel the specific composition of the histotroph and to elucidate the gene expression signature of both embryo and maternal endometrium during diapause. Tandem mass spectrometry, RNA sequencing and confocal microscopy complemented by *in vitro* embryo culture were applied. First results indicate that the embryo undergoes an inherent discontinuity of development already following fertilization. During diapause and, most strikingly, prior to elongation, remarkable changes of both the embryo and the histotroph occur. Thereafter, embryo elongation and implantation take place at normal proliferation rate, thus marking the escape from the diapause phase. Disentangling the recent molecular findings in a strategic way will allow to (a) decipher the underlying cues in roe deer and (b) compare across species including rodents, marsupials and mustelids, where current molecular knowledge is advance, to unravel conserved mechanisms across species. The benefits of this research are on hand: reproductive principles in roe deer may unravel novel strategies of population management. Knowledge about molecular mechanisms of developmental arrest is moreover promising for use during *in vitro* embryo production in further species including livestock, wildlife and human, for studying cell proliferation inhibition to extend pluripotency, to arrest senescence, and to suppress cancer proliferation.

## Is the diapause relevant for conservation of endangered species?

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The biological phenomenon of delayed embryonic implantation, also called diapause, occurs in over 130 mammalian species. However, the regulation pattern of embryonic dormancy prior to implantation can be quite different. Only few major elements of these complex regulatory cascades have been elucidated yet. The presentation has therefore a more hypothetical approach by discussing, in the first part, how to determine the optimal timing and conditions for the transfer of in vitro derived embryos in species such as roe deer and giant panda. In the second part, delayed implantation and its spectrum of regulatory mechanisms will be evaluated in regards to future use as tool for long-term embryo storage above 32 degrees Fahrenheit.

## Giant panda ex-situ conservation in the Chengdu PandaBase and research progress on embryonic diapause

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As a world-renowned center, the Chengdu Research Base for Giant Panda Breeding focuses on conserving and breeding giant pandas and other endangered wildlife. We have bred 284 panda cubs and established the world's largest self-sustaining captive bred population. Assisted reproduction and breeding, conservation genetics, habitat conservation and management of the giant panda national park and the prevention and control of the major epidemic wildlife diseases are the four main research foci of the Research Center of the Panda Base. Studies on fetal development are important for understanding reproduction in giant pandas. However, many questions remain unanswered representing a challenge for the ex-situ conservation. Breeding records from Panda Base indicate that the length of gestation in the giant panda can vary between 83 and 200 days. This suggests delayed implantation during pregnancy in the giant panda. In 1984, Hodges further proved delayed implantation occurred in giant pandas based on the measurement of pregnanediol-3 $\alpha$ -glucuronide. Zhang et al. showed that delayed implantation increases flexibility in the timing of birth but is not important in dictating infant growth. Here, we want to make a comprehensive introduction from the hypothesis of the diapause of giant panda embryos and its further confirmation, as well as the related endocrine hormone study on giant panda breeding including estrogen-progestin luteinizing hormone and related urine metabolites 13,14, dihydro-15-ketoprostaglandin F $2\alpha$  (PGFM).

## Unravelling the giant panda reproductive biology: diapause and (pseudo)pregnancy

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The mechanism behind diapause remains undefined in giant pandas (GPs) and its unravelling is, besides lacking access to appropriate sample matrices, additionally compromised by the occurrence of pseudopregnancy. The latter condition is not only mimicking the high-level progesterone profile during pregnancy, representing the active luteal phase after corpus luteum (CL) reactivation, but also includes a CL dormancy phase after ovulation showing a similar modestly increased progesterone profile as observed with diapause. The signals involved in regulating this obligatory biphasic progesterone secretion are not known. Diagnosing conception and pregnancy is therefore challenging, as well as defining the exact length of a GP pregnancy after blastocyst reactivation. Our project aims the unravelling of the GP reproductive biology, with the focus of this study on defining pregnancy length and investigating potential mechanisms behind reactivation and consequent attachment, based on conventional endocrine monitoring with immunoassays (estrogen, progesterone and 13,14-dihydro-15-keto-PGF<sub>2α</sub> (PGFM)) supported by targeted and untargeted UHPLC-HRMS. Four GPs (12 cycles; 2 bred-birth, 7 bred-non-birth, 3 non-bred) were included pursuing full-cycle daily urine samples for full-profile endocrine monitoring. A prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)-immunoassay was performed on 3 GPs' active luteal phase samples (5 cycles; 2 bred-birth, 3 non-birth; n=85), with parallel screening for prostaglandins and metabolites using UHPLC-HRMS. Normalisation of urinary concentrations was done with urinary specific gravity.

We confirmed no significant differences in the progesterone/PGFM profile between pregnant, potentially pregnant and pseudopregnant GPs until the first PGFM peak, indicating a pre-programmed endocrine metabolism after ovulation, regardless of conception. Nevertheless, we were able to define pregnancy length based on sequences in the progesterone profile, leading to a 37-days lasting pregnancy after CL reactivation, the latter characterized by a rapid increase in progesterone. Initial changes in PGFM were observed 13 days after CL reactivation, matching with the window of attachment in the mink-model. PGE<sub>2</sub> significantly increased from baseline prior to changes in PGFM. This suggests a potential role of PGE<sub>2</sub> as luteotropin in CL reactivation, as seen in dogs during the first third of (pseudo)pregnancy. The PGE<sub>2</sub> signal is likely contributing to the PGFM peak (cross-reactivity). After the PGFM peak, in birth-cycles an average 3.63-fold increase, compared to 1.32 in non-births, was observed. The higher PGE<sub>2</sub> levels in bred cycles after presumed attachment also indicate a potential role for PGE<sub>2</sub> in attachment/embryonal development. The PGE<sub>2</sub> concentrations were generally low (pg<sub>PGE<sub>2</sub></sub> versus ng/mL<sub>PGFM</sub>) because of the known rapid metabolism of eicosanoids, but screening with UHPLC-HRMS confirmed the presence of PGE<sub>2</sub>-metabolites.

## Measurement of urinary estrogens and cortisol in the giant panda during delayed implantation and blastocyst reactivation

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Giant pandas are seasonal breeders with a single Spring oestrus. Following successful conception, there is a biphasic luteal phase including a variable-length embryonic diapause and a short period of fetal development before birth. Pandas undergo pseudopregnancy, with similar behaviours and progesterone (P4) profiles in both pregnant and non-pregnant pandas. At 24 days before the end of the cycle/birth, all pandas indicate the start of a urinary 13,14-dihydro-15-keto-PGF<sub>2α</sub> (PGFM) spike – this is a known fixed point in the cycle, and the peak of the spike occurs coincidentally with peak P4 concentrations. Currently there are no confirmatory non-invasive biomarkers of conception, blastocyst reactivation, implantation or pregnancy in the giant panda. The duration of fetal development is uncertain, however the consensus is that it is less than 40 days.

We hypothesised that steroid hormones are involved in blastocyst reactivation and that changes in the urinary concentrations could be non-invasive biomarkers of implantation in the panda.

We monitored urinary estrogens and cortisol across the final 50 days of the oestrous cycle in 13 cycles from 5 female pandas. This represented 4 births, 4 non-bred cycles, and 5 non-birth cycles. Hormone profiles of P4 and PGFM were also evaluated to determine the switch into the active luteal phase and to indicate the cycle end date. Hormones were measured by immunoassay, and concentrations were corrected for urinary specific gravity.

Urinary estrogens did not show a consistently low concentration as previously reported in the literature. We identified an increasing profile at the end of putative diapause, increasing 2-fold for 16 days before the start of the PGFM spike, and increasing a total of 2.6-fold until 9 days after the start of the PGFM spike. This shift in the profile begins 41 days before the end of the cycle/birth, and occurred in all reproductive outcomes. Cortisol profiles generally showed low concentrations with minimal fluctuations at the end of the diapause period, however differences were observed between reproductive outcomes. Females who gave birth showed fluctuations beginning at -10/-11 days prior to the start of the PGFM spike, which continued in an increasing trend across the PGFM spike and fetal development. Non-bred females showed fluctuations prior to -10/-11 days from the start of the PGFM spike which ceased a week before the start of the PGFM spike. Non-birth profiles showed a combination of features from both the birth and non-bred profiles, but no cycle showed a profile alike birth.

Concluding, blastocyst reactivation and implantation in the giant panda is associated with a period of increased estrogens. This appears to be maternally driven with no influence of any early pregnancy, thus at this stage is not a marker of success. Cortisol, however, is suggested to be influenced by early pregnancy, therefore monitoring the profile across the luteal phase has the potential as a predictive biomarker of implantation and pregnancy success in the giant panda.

## Embryonic diapause in carnivores: the American mink (*Neovison vison*) as a model species

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Embryonic diapause uncouples mating from parturition and allows both to occur when conditions are most favorable for reproductive success and offspring survival. Many carnivores display a condition of obligate diapause, i.e., occurring in every gestation. During diapause, development of the embryo is arrested for a species-dependent duration. Seasonal cues regulate the induction of and escape from diapause in these species. Diurnal rhythms of melatonin translate photoperiod into a regulatory signal. In the mink model, the melatonin secretion patterns regulate the secretion of pituitary prolactin, such that prolactin levels are suppressed during the short days, maintaining diapause. After the vernal equinox, daylight during a photosensitive period (from 12-15 h after dawn) reduces the daily duration of melatonin synthesis and permits prolactin secretion. This results in re-initiation of embryonic development, followed by implantation and the completion of gestation. During diapause, the corpus luteum produces only low levels of progesterone. It is rapidly reactivated by the seasonal surge in prolactin. Studies of luteal function in the mink have shown that prolactin upregulates its own receptor, along with that of luteinizing hormone, and key steroidogenic enzymes essential for progesterone secretion. In vivo treatment of mink with prolactin during diapause causes luteal reactivation, progesterone secretion, and rapid termination of diapause. Expansion of the embryo, protein synthesis and cell proliferation occur within 48 h of initiation of treatment with exogenous prolactin, and embryos implant within 11-12 days. Reciprocal transplants of mink blastocysts with those of the ferret, a non-diapause species, revealed that the proximal control of diapause resides in the uterus. While comparative analysis indicates that some mechanisms of uterine regulation are conserved across taxa, the rodent paradigm of termination of diapause by progesterone treatment followed by an estrogen injection fails in the mink, due to lack of estrogen receptors in the uterus during diapause. In contrast, prolactin receptors are present in the mink uterus. By global gene expression analysis we have shown that in vivo prolactin treatment modifies the transcriptome of the uterus during escape from diapause. With differential expression of more than 350 genes. Among these is a cluster of genes that regulate the abundance of polyamines in the uterus, providing clues about the uterine signal that induces embryo reactivation. Recent investigations have shown that prolactin, acting through its cognate receptor, promotes polyamine synthesis in the uterus. In overview, carnivore diapause is regulated by photoperiod, melatonin, pituitary prolactin, ovarian steroids, and uterine factors, including polyamines.

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## Evaluating mink fecal proteins during embryonic diapause and placental pregnancy for non-invasive pregnancy diagnosis

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Currently, there is no method to diagnose pregnancy non-invasively in most wildlife species that experience delayed implantation and pseudopregnancy, either during embryonic diapause or placental pregnancy. For animals maintained in zoos, the ability to detect and monitor gestation is valuable for management purposes and may help identify the point of reproductive failure. There is growing interest in characterizing fecal proteins that may be indicative of pregnancy, but performing controlled biomarker discovery studies on zoo-housed animals is confounded by inherent challenges associated with heterogenous populations and varying husbandry practices among institutions. Furthermore, there is no traditional model species that naturally undergoes both delayed implantation and pseudopregnancy. Our aim was to use farm-raised mink (*Neovison vison*) as a model species to evaluate changes in the fecal proteome associated with pregnancy. Specific objectives were to: 1) determine if peptides or peptide fragments were different in the feces of parturient versus non-parturient mink and; 2) identify the proteins of interest.

Fecal samples (n=12) were collected from black color phase mink (n=6) maintained on a commercial farm (Wisconsin, USA). Samples were selected retrospectively from parturient (P; n=3) and non-parturient (NP; n=3) individuals and were collected at two timepoints: during embryonic diapause (ED) and 21 days prepartum (PP) from P females, or; at parallel timepoints for NP females. Total protein was extracted from each sample and two-dimensional differential in-gel electrophoresis was utilized to separate proteins and assess differences in protein spot abundance among samples. Spots meeting specific criteria (student's t-test between groups;  $P < 0.10$ ;  $> 2.5$  fold change) were selected for matrix-assisted laser desorption/ionization- time-of-flight and protein identities were ascertained by peptide mass fingerprinting. Candidates with a total ion confidence interval of greater than 97.0% were accepted as positive identification.

The number of spots that resolved on each gel ranged from 1874 to 2302 (mean of  $2107 \pm \text{SEM } 62.2$ ). Protein identities were obtained for 15 individual spots. During ED, six spots (angiotensin-converting enzyme 2, interleukin-36 receptor antagonist, carboxypeptidase A1 (two spots), carboxypeptidase A2, chymotrypsin-like protease CTRL-1) were higher in P vs. NP and one spot (intestinal fatty acid-binding protein) was higher in NP. At PP, seven spots (cytosol aminopeptidase (three spots), calcium-activated chloride channel regulator 1, carboxypeptidase A1 (two spots), chymotrypsin) were higher in P and two (ovalbumin, protein PRR14L) were higher in NP. Several of these proteins hold functions integral to pregnancy establishment or maintenance and may be useful in diagnosing and monitoring pregnancy as well as providing insight into processes responsible for reproductive failure. This is the first report describing changes in the mink fecal proteome as it relates to pregnancy and the first description of changes in specific fecal proteins during embryonic diapause in any species.

## Putting pregnancy on hold: three species, two continents and one hypothesis

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Embryonic diapause was first identified in 1854. Since then major advances have been made in understanding the external influences that control diapause and how that translates to hormonal control of the uterus. However, important questions remain. Primarily, how does the uterus convey this information to the embryo? Current evidence suggests that the regulation of diapause is mediated by components of the uterine secretions rather than direct effects of ovarian hormones on the blastocyst. However, the identity of the essential signalling molecule(s) is unknown. Secondly, how does the embryo maintain viability during this extended period of quiescence? In the majority of other cell types, induction of cell cycle arrest is a precursor to apoptosis, senescence or terminal differentiation. Thirdly, are the mechanisms involved conserved across species? Over 130 mammalian species in 9 different orders undergo embryonic diapause worldwide. Not surprisingly, the selective pressures on species with different life-histories have led to the evolution of diverse environmental signals and hormonal pathways that regulate diapause. However, despite the varying mechanisms regulating uterine quiescence and reactivation, the signalling mechanisms between the uterus and the embryo that control entry into, maintenance of, and reactivation from diapause appear to have been conserved, even amongst non-related orders. The first evidence for this came from a study showing that the transcription factor MSX is expressed in the uterus during diapause and not during reactivation in three distantly related species; the mouse *Mus musculus*, the mink, *Neovison vison*, and the wallaby, *Macropus eugenii*. These three species are the most extensively studied mammalian diapause species. Despite many differences with regards to their external and hormonal control of diapause between these three species, we have now found conservation of a number of other molecular factors around diapause and reactivation. In particular, the Epidermal growth factor (EGF) family and the polyamines both appear to have a conserved role in reactivation from diapause. Inhibition of polyamines causes entry into diapause in both the mink and mouse and polyamines are able to induce reactivation of the diapause blastocyst *in vitro* in the mink. However, we have also now shown reactivation of the mink diapause blastocyst *in vitro* in the absence of polyamine supplementation. Thus, although the signalling mechanisms via which the uterus is induced into diapause vary amongst species, the molecular communication that occurs between the uterus and the embryo to control diapause is conserved implicating a universal mechanism for maintaining embryo health amongst all mammals.

## If all Pinniped species are supposed to have embryonic diapause, then why might not this one?

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All pinnipeds are presumed to have an obligate embryonic diapause that stretches the reproductive cycle such that it fits in one full calendar year and ensures that energetically-costly life history events occur during benign ambient conditions. Moreover, the partitioning of diapause and active gestation ultimately dictates how energy is allocated across pregnancy. Among pinniped species, embryonic diapause can range from a few weeks to >4 months, which raises the question: 'What determines the ideal balance of how long embryonic diapause versus active gestation should be, in order to maximize fitness?' To answer this question, we have used the Weddell seal (*Leptonychotes weddellii*) as a model study species, due to their highly-seasonal environment and long-term (50-yr) demographic study in Erebus Bay, Antarctica. Here I pair early pregnancy diagnosis via ultrasound (embryos > 3 mm diameter) with a suite of molecular "-omic", physiological, and behavioral tools to characterize the Weddell seal's reproductive phenology and elucidate factors that promote positive reproductive outcomes. Calculated embryonic growth curves revealed that the Weddell seal has the shortest embryonic diapause of any pinniped, or may be the first seal species identified to have lost embryonic diapause from the reproductive cycle altogether. Because embryonic diapause is shorter in duration (<2 weeks), the Weddell seal instead has a protracted active gestation of >10.5 months. Despite significantly increasing dive efforts, Weddell seals gain less mass across gestation as compared to other pinnipeds. Thus, extending the duration of fetal development may serve to lower the daily energetic costs of active gestation. Our integrative approach spanning across multiple levels of organization demonstrates that energetic constraints have shaped the life history of this polar marine mammal. This has important implications for population dynamics and conservation, and is integral to anticipating how top predators will respond to a changing world.

## Postimplantation delayed development in Seba's short-tailed fruit bat, *Carollia perspicillata*

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Pregnancy has been studied in fruit bats, *Carollia perspicillata*, maintained under controlled conditions in captivity or collected from a reproductively synchronized wild population on the Caribbean island of Trinidad. Gestation periods in wild-caught bats bred during their first year in captivity exhibited remarkable variation (105-178 days). This was lessened in wild-caught bats bred during their second year in captivity. It was concluded that this variability must have been caused by stress, as most captive-reared and -bred bats exhibited gestation periods of 113-119 days (= the normal/non-delayed gestation length for *Carollia*), while one such group inadvertently subjected to significant stress exhibited greater variation in gestation length (113-141 days). Histological studies established that the embryonic diapause occurs after implantation, at the primitive streak stage. In the wild, *Carollia* exhibits two, synchronized reproductive periods. The first pregnancy includes a minimal delay period estimated to be at least 44-50 days, but possibly longer. The adaptive significance of this may be that it permits the females to eventually give birth, have a postpartum estrus, and establish new pregnancies at a more propitious time of year (i.e., later in a long dry season). Pregnancies established during the second reproductive period normally proceed without significant delays. It seems unlikely that delays in the first pregnancy are triggered by stress. Rather, they may have evolved to lessen challenges faced by the animals many months later, when they would be simultaneously pregnant and lactating. Comparative immunocytochemical and ultrastructural studies of normal vs delayed pregnancies strongly suggest that inadequate trophoblastic differentiation within the developing chorioallantoic placenta may play a central role in retarding embryonic growth. During delay, much of the cytotrophoblast in the developing placenta appeared notably undifferentiated, and the placenta had been invaded only to a limited extent on its embryonic side by mesoderm. In contrast, in placentae serving post-delay somite and limb bud stage embryos, areas of highly differentiated syncytiotrophoblast perforated by maternal vascular spaces (trophospongium) had also formed. First contact of the allantois with the developing placenta was noted at the somite stage. This initiated eventually widespread invasion of the trophospongium by more differentiated cytotrophoblast, mesenchyme and allantoic blood vessels. The observed pattern of morphogenetic events suggests that this differentiated cytotrophoblast may promote mesenchymal invasion and angiogenesis on the embryonic side of the placenta, thereby stimulating renewed embryonic development. The diapause occurring in *Carollia* is notable for being postimplantational. There appears to be good reason for this. Early development proceeds to the expanded, zona-free blastocyst stage in one of the oviducts. Implantation is then initiated soon after blastocyst entry into the uterus and relatively close to the uterotubal junction. This always leads to centering of the chorioallantoic placenta in the fundic region of *Carollia*'s simplex uterus and ultimately to equal vascularization of the placenta by both uteroovarian arteries. This rich vascularization of the placenta presumably permits the eventual production of a single, very large, and highly precocious infant.

## Energy utilization by the diapausing embryo in mouse

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Lipid Droplets (LDs) are the intracellular sites for the storage of triglycerides, steryl esters and retinyl esters, critical for cellular metabolism, energy homeostasis and signalling. High amounts of LDs in mammalian eggs are maintained without marked signs of their utilization throughout embryo development until implantation. ATP requirements of cleaving embryo are met mainly by the utilization of carbohydrates, so even if deprived of LDs, the embryo develops normally. Limited utilization of energy resources during preimplantation development is because the division of embryonic cells (blastomeres) is lacking S-phase. Following each cleavage, blastomeres get half of their original size until reaching the stage of blastocyst. At this stage the embryo starts its growth and establishes contact with the uterus by implantation and after that, starts to utilize maternal nutritional resources. What is the role of energy resources so generously distributed in form of LDs in mammalian egg? Are LDs stored in the egg for some emergency situations which the embryo may face during its preimplantation development? We hypothesised that LDs have the crucial role in the maintenance of Embryonic Diapause (ED), a temporary arrest of the embryo while waiting for the maternal receptivity to implant. To verify this, following natural mating of mice the ovariectomy has been performed in females at 2.5 day post coitum (dpc) to avoid embryo implantation. Then, diapausing blastocysts were flushed from the uterus at 6, 8, 10, 12 dpc. Non-diapausing control blastocysts were collected at 4 dpc. Collected embryos were subjected to RNA sequencing, transmission electron microscopy and lipid imaging analysis. We show that LDs are utilized during the progression of ED in mouse. Diapausing blastocysts remained metabolically active. Breakdown of LDs occurred by autophagy, which is particularly elevated during ED. During first 3 days of diapause, energy was utilized by the embryo to continue its growth until reaching about 200 cells, and for biogenesis and transport of exosomes and multivesicular bodies. In subsequent days, diapausing embryos have arrested their growth and were releasing exosomes intensively. We further showed that the amount of LDs in fully grown oocytes from various mammals is correlated with their species-specific length of diapause. This study uncovers the role of LDs storage in mammalian egg.

## The critical role of energy availability in embryonic diapause in *Cynopterus sphinx*

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The bat, *Cynopterus sphinx*, exhibits two successive pregnancies in a year however; its gestation length varies significantly in the two successive pregnancies; the prolonged gestation period during the winter pregnancy is due to delayed embryonic development where the embryo is arrested in the gastrula stage. The period of embryonic diapause corresponds with the period of adverse scarcity of food, winter dormancy, accumulation of white adipose tissue well as decreased serum progesterone level. Thus the aim of current study was to find out the mechanism by which adverse environmental condition (accumulation of white adipose tissue) causes impaired progesterone synthesis and embryonic diapause. The study showed a significant increase in circulating leptin level and significant decrease of serum adiponectin expression during the period of increased fat accumulation, which coincided with significant decrease in serum progesterone level and delayed embryonic development in *C. sphinx*. The study showed increased leptin receptor and decreased expression of adiponectin receptor in the corpus luteum and in the utero-embryonic unit during the period of delayed embryonic development. The results further showed increased circulating levels of glucose and insulin and increased expression of insulin receptor (IR) and glucose transporter (GLUT) 4 proteins in the adipose tissue during winter. However, there was decreased expression of GLUT 3, 4 and 8 and IR proteins in the ovary and utero-embryonic unit during the embryonic diapause, suggesting that increased leptin and decreased adiponectin might be responsible for decreased availability of glucose for the utero-embryonic unit and increased glucose availability for the adipose tissue; and simultaneously suppressing the progesterone synthesis and embryonic diapause. To validate this, both *in vitro* and *in vivo* approach was taken. The bats were treated exogenously with adiponectin during the period of embryonic diapause, and it showed reactivation of luteal steroidogenesis and embryonic development by promoting increased uptake of glucose, which consequently stimulated angiogenesis and cell proliferation/survival. The *in vitro* study showed suppressive effect of leptin on progesterone synthesis. The effect of high dose of leptin on ovarian steroidogenesis was found to be mediated through decreased expression of StAR and LH-R proteins in the ovary. To conclude it is observed that both fat accumulation and embryonic development are energy requiring processes which utilizes glucose which in winter is mainly transported to adipose tissue, diverting glucose away from utero-embryonic unit in *C. sphinx* during the period of embryonic diapause. Leptin and adiponectin thus provides the link between low energy status and embryonic diapause during the period of adverse environmental condition in *C. sphinx*.

## **Characterization of diapause and ovarian reactivation by non-invasive fecal progesterin analysis in the fisher (*Pekania pennant*) and wolverine (*Gulo gulo*)**

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Several seasonally-breeding species within the Family Mustelidae undergo a period of embryonic diapause, followed by ovarian reactivation and subsequent embryo implantation and development. With the exception of the American mink, the duration of diapause and possible triggers for ovarian reactivation are poorly understood in mustelids. For fishers and wolverines, their highly secretive nature and preference for remote habitats have made longitudinal monitoring of reproductive hormonal patterns difficult to conduct in wild populations. In this preliminary study, fecal samples were collected non-invasively from two US zoo-housed females (n=1 fisher, 153 samples, Northwest Trek Wildlife Park; n=1 wolverine, 237 samples, Detroit Zoo) for 20 to 22 consecutive months while paired with males for potential breeding. Progesterone metabolites were measured in extracted fecal samples using validated enzyme immunoassays. Temporal changes in progesterin levels in both females suggested occurrence of three distinct hormonal phases: a basal (non-reproductive) phase, a post-ovulation (diapause) phase and a reactivation (gestational) phase. The duration of each phase was calculated using an iterative process, with the beginning and end of each phase delimited by three or more consecutive samples with low, intermediate or elevated values. For each phase, values were averaged and reported as mean $\pm$ SEM. In the fisher, the basal phase (i.e., after parturition/end of pseudopregnancy but prior to ovulation) was characterized by progesterin levels of  $821.6 \pm 81.5$  ng/g dried feces, followed by a post-ovulation phase ( $4149.4 \pm 255.3$  ng/g) lasting 287 days, and a reactivation phase ( $9151.9 \pm 398.2$  ng/g) occurring for 65 days. In the wolverine, progesterin levels during the basal period averaged  $1016.4 \pm 126.7$  ng/g dried feces, followed by a post-ovulation phase ( $4896.0 \pm 359.8$  ng/g) lasting 157 days, and a reactivation phase ( $21589.1 \pm 1399.0$  ng/g) of 86 days. For both females differences in progesterin levels between all three phases were highly significant ( $p < 0.01$ ). Because neither female was observed to give birth, it was assumed that both females experienced pseudopregnancies. These preliminary data in a fisher and wolverine suggest that fecal progesterin analysis may be of value for assessing the occurrence of ovulation, diapause and ovarian reactivation in both species; however, differentiation between pregnancy and pseudopregnancy may not be possible. Further, assessment of fecal progesterin patterns relative to seasonal benchmarks, such as the winter solstice, may offer insight into possible triggers (eg., daily light exposure) for ovarian reactivation and implantation in both species. Finally, as previously demonstrated in our research with North American river otters, longitudinal fecal progesterin monitoring may allow prediction of parturition timing in these two species, providing an important tool for captive breeding and conservation efforts.

## Chromatin regulation by growth, in proliferation and in pause

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Mammalian development starts with fertilization and the first cell fate decisions are made before the embryo implants into the uterus. Pluripotent cells arise within the blastocyst stage embryo, have the potential to give rise to all cell types in the body, and can be captured indefinitely in vitro by propagating the inner cell mass of the blastocyst under self-renewal conditions. Importantly however, pluripotency is transient in nature, and only exists for about three days during mouse development. The exception to this rule is embryonic diapause, a state where pluripotent cells remain dormant for extended periods of time. Regulation of embryonic diapause at the molecular level remains elusive. We have recently shown that partial inhibition of the growth regulator mTor induces developmental pausing of mouse blastocysts ex vivo and of ES cells in vitro. mTOR-inhibited blastocysts reside in a transcriptionally, translationally and metabolically suppressed state similar to in vivo diapaused embryos. Here I will discuss chromatin and RNA-based mechanisms which regulate developmental pausing in the mouse.

## LIF a multifunctional factor regulating blastocyst implantation and viability during diapause

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In mammals, uterine preparation for embryo implantation is primarily regulated by the ovarian steroid hormones estrogen and progesterone. These initiate a variety of cellular changes in the uterus by the autocrine and paracrine expression of many growth factors and cytokines. In rodents and some other mammals, the key factor governing embryo implantation is Leukemia Inhibitory Factor (LIF). In vivo, LIF is transiently expressed by the uterine endometrial glands during pregnancy prior to implantation. LIF acts on the uterine luminal epithelium (LE) converting it from a non-responsive to an embryo responsive epithelium. Deficiency of LIF results in implantation failure that can be rescued by a single injection of LIF. However, in a uterus under the influence of progesterone, lack of LIF results in blastocysts entering diapause, which can then be disrupted by LIF which leads to implantation. LIF's effects on the uterus are mediated through the LE as the LE expresses the LIF receptor complex. Upon stimulation by LIF, the STAT3 and MEK/MAP kinase pathways are activated in the LE. To identify the changes LIF induces in the LE so initiating blastocyst implantation, we analyzed gene expression and annotated signaling pathways that are induced in the LE at different timepoints within the first 6 hours of LIF expression. At least 18 signaling pathways and some 4000 genes are induced/repressed within the first 6 hours revealing the molecular complexity of how an epithelium responds to an inductive signal.

Intriguingly, the reproductive requirement for LIF was first shown by its role in preventing embryonic stem (ES) cell differentiation during culture. ES cells are derived from the inner cell mass (ICM) of the blastocyst and blastocysts in diapause require LIF signaling to sustain an ICM. LIF therefore has 2 key roles in regulating murine diapause. The first is determining uterine receptivity at initiating embryo implantation and secondly as a survival factor to sustain the ICM during prolonged diapause. How embryo implantation evolved resulting in LIF having these 2 separate roles is still a mystery.

## **Proteomic and transcriptomic analysis reveals dynamic molecular changes governing embryonic diapause and reactivation for implantation in mice**

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Implantation of the blastocyst into uterus is the gateway for further embryonic development in mammals. Programming of blastocyst to an implantation-competent state known as blastocyst activation is the determining factor for implantation into the receptive uterus. However, it remains largely unclear how the blastocyst is globally programmed for implantation. Employing a delayed implantation mouse model and omics approach, we show that the blastocyst undergoes extensively programming both at the transcriptional and translational level essential for implantation. For the proteomic analysis, a total of 2255 proteins were detected. Various cellular and molecular processes, including protein translation, aerobic glycolysis, pentose phosphate pathway, purine nucleotide biosynthesis, glutathione metabolism, and chromatin organization were identified as differentially regulated, which was also observed at the transcription level and consistent with the previous research. We demonstrated a remarkable activation of mitochondria in blastocysts upon reactivation from dormancy, highlighting their essential physiological significance. Moreover, the activities of the endosome–lysosome system were prominently enhanced and the cell-cell tight junctions and extracellular matrices (ECM) were weakened in the mural trophoblast of reactivated blastocysts, accompanied by active phagocytosis at the fetal-maternal interface. Through analyzing the transcriptional changes, we also demonstrate that reactivation of X chromosome, one of the most important events during peri-implantation female embryonic development, is not completed even in blastocysts under conditions of dormancy, despite long-term of suspension in the uterus. For the embryo derived signal attending the crosstalk between the blastocyst and the uterus, the differentially expressed profile of secretory proteins suggested that the blastocyst functions as a pro-inflammatory body to secrete pro-inflammatory signals, such as TNF $\alpha$  and S100A9, thereby triggering embryo-uterine attachment reaction during implantation. Utilizing multiple approaches, including the protein soaked bead transfer model and uterine gene deletion model, it was proven that embryo derived signaling function in both autocrine and paracrine manners to induce the inflammatory-like response during the implantation. Collectively, our data systematically and comprehensively disclose the programming of blastocyst reactivation from diapause for implantation and uncover previously undefined roles of blastocyst during implantation.

## Dormancy progression in mouse embryonic diapause

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We studied the process of how mouse embryos enter a dormancy status at a cellular level. Immunofluorescent analysis of differentiation markers for epiblast, primitive endoderm and trophoctoderm suggested that cell differentiation status was maintained during 7 days in diapause. To understand the progression of cellular dormancy during diapause, we examined the expression of a transgenic cell cycle marker Fucci2 and Ki67 by antibody staining, in addition to direct counting of nuclei in embryos. From these analyses, embryos during diapause were categorized into four stages by cell number and cell cycle. Cell cycle arrest occurred from the ab-embryonic region, and from the TE to the ICM in the embryonic side.

We also observed cell cycle transition by live imaging of Fucci2 embryos during the reactivation in culture from dormant status. Cell cycle activity was initially recovered from the embryonic side of embryos and eventually spread throughout the whole embryo. We also found that embryos in later stages of diapause required a longer period of time for reactivation.

From these observations, it was shown that entrance into and exit from dormant status varied depending on cell types and location of cells in an embryo. These results suggest that embryonic diapause includes multiple steps and the mechanisms involved in cellular dormancy may be distinct between embryonic regions.

## Coming unstuck gracefully: how uterine epithelial cells let blastocysts in

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In species which have hemochorial placentation such as rats and humans, where the blastocyst deeply invades the endometrium, a key step is breaching of the maternal epithelium to allow access to maternal blood. Breaching an intact epithelium from the apical surface is a highly atypical phenomenon, because epithelial cells are designed to form a barrier which is not intended to be breached. To enable this barrier function, epithelial cells have special junctions on their lateral and basal surfaces which are plasma membrane structures. Thus, for a blastocyst to begin pregnancy, this barrier must be breached and these specialised junctions must be altered or downregulated.

The principle epithelial cell junctions are the tight junction, adherens junction and desmosomes of the lateral plasma membrane and the hemidesmosome and focal adhesion of the basal plasma membrane.

The tight junction is the apical-most junction and is a 'seal' to prevent passage of molecules between epithelial cells. In rats and humans as uterine receptivity approaches, the tight junction extends further down the lateral plasma membrane and becomes more geometrically complicated. This indicates that the tight junction reduces paracellular transport and more closely regulates the contents of the uterine lumen for blastocyst development. Concomitantly, water transport molecules, aquaporins, show a shift to transcellular transport just as paracellular transport is reduced. Immediately below the tight junction is the adherens junction which holds uterine (and other) epithelial cells together. In the uterus, as the tight junction extends further down the lateral membrane, the adherens junction, which is very evident early in pregnancy, progressively breaks down until by blastocyst attachment this junction is no longer visible. Similarly, the terminal web, a key band of actin filaments in epithelial cells involved in cellular adhesion which joins with the adherens junction to support the apex of epithelial cells, also disappears by the time of implantation. The other major junction on the lateral plasma membrane is the desmosome, referred to as a 'spot weld', which holds epithelial cells together. As pregnancy progresses these junctions are more than halved in number and the reduction is all along the lateral plasma membrane. These observations are compelling evidence that uterine epithelial cells become progressively less adherent to each other as implantation approaches. On the basal plasma membrane, junctions hold uterine epithelial cells to the underlying connective tissue. Ultrastructural hemidesmosomes are lost during early pregnancy and we also know that there is a very large downregulation of the key basal adhesion molecules, paxillin and talin, which are components of the focal adhesions also holding uterine epithelial cells to the connective tissue.

These observations show that all uterine epithelial junctions which hold the cells to each other or to the underlying connective tissue are downregulated by the time of uterine receptivity. This ordered restructuring of junctions and their molecular assemblies is a device to allow penetration of the maternal epithelium, and is a component of the wider reorganization of the plasma membrane of uterine epithelial cells in preparation for receptivity which is collectively known as the 'plasma membrane transformation' (PMT). This broader PMT however, occurs even in species without hemochorial placentation, suggesting that common structures and molecules are involved at the earliest stage of placentation across species.

## Transcriptome profile during diapause of mouse embryos

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The Embryonic Diapause (ED) is the state of the temporary suspension of the embryo development at the blastocyst stage in circumstances of lack of uterine receptivity signal. The period of “waiting” of developed mouse blastocysts for the implantation signal can extend from few hours to several days. The transcriptome changes governing the adaptation of the blastocyst to the diapause remained largely unknown. Here, we show the transcriptome profiling of the blastocysts during different time points of ED (6.5, 8.5 and 12.5 days post coitum), introduced by ovariectomy. A total of 3445 genes were differently expressed between control (4.5 dpc) vs advanced (12.5 dpc) time point of ED. Various cellular and molecular processes, including pathways involved in mitotic cell division, cellular adhesion, transport along microtubules or vacuole organization were identified as differentially regulated during the course of ED. Except those mentioned, the most up-regulated pathways were the pathways involved in cell survival during starvation period; catabolic process, cellular lipid catabolic process, branched chain amino acid degradation and autophagy. The results provide evidence that blastocysts at various stages of the diapause are molecularly distinguishable in a global transcription profile. This study has identified molecular pathways that can explain many of the mechanisms underlying the blastocyst's ability to survive a long pre-implantation period. This results shed the new light on the molecular dynamics of the embryo during diapause.

## Transcriptome analysis of embryonic diapause in the tammar wallaby

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The tammar wallaby is the best characterised model for marsupial embryonic diapause, displaying both lactational and seasonal control and highly synchronised reactivation around the summer solstice. During diapause, the unilaminar blastocyst remains unexpanded and has around 80 morphologically similar cells. About five days after removal of pouch young (RPY) during lactational diapause, a progesterone pulse from the corpus luteum induces reactivation of the blastocyst and it begins to expand. To investigate molecular changes associated with reactivation of diapause, we compared blastocyst and endometrium transcriptomes during lactational diapause and 5–9 days after RPY. Gene ontology (GO) enrichment analysis indicated down-regulation of genes involved in the G1/S transition of the cell cycle in blastocysts during early reactivation. This contrasts with the mouse, in which G1/S transition genes were previously shown to be up-regulated after reactivation from diapause, suggesting that fundamental differences exist between marsupials and eutherians in the cellular mechanisms of cellular quiescence during diapause.

## Dynamic transcriptome changes during embryonic diapause and reactivation in the embryo and endometrial epithelium of the European roe deer (*Capreolus capreolus*)

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The European roe deer (*Capreolus capreolus*) pre-implantation embryo development is characterised by a four month period of embryonic diapause, after which the embryo rapidly elongates and implants. We found that roe deer pre-elongation developmental pace is 10-times slower than in cattle. In roe deer, endometrial secretions at implantation are 1.5-fold higher than during diapause, and morphological changes of the embryo coincide with changes of the uterine fluid composition. As the reactivation regulatory mechanism are still unknown, we analysed the embryonic and endometrial transcriptome changes during diapause and reactivation. Samples were collected at regular huntings between September and January 2015-2017. A total of 360 animals was sampled and 537 pre-implantation embryos were collected (77% recovery rate). An additional group of six day 14 ex vivo flushed embryos were included to represent early blastocysts from captured roe deer at IZW Berlin. Embryonic DNA was extracted for the determination of the number of embryonic cells. Total RNA from 87 embryos and endometrial luminal epithelial (LE) cells RNA from 56 different females, covering the period of embryonic diapause and reactivation, was subjected to RNA-seq. Raw sequence reads were analysed using a customised Galaxy pipeline. A pseudotime analysis (CellTree) was performed to gain insight into the transcriptomic variation of diapausing embryos, in which DNA content was used as proxy for developmental progression. Differentially expressed transcripts (DET) were identified in a time-course dependent manner with the ImpulseDE2 algorithm. To elucidate dynamic changes, a self-organising tree algorithm (SOTA) was used. Gene ontology (GO) and pathway analyses were conducted with the DAVID tool. As determined by a rise in DNA content, embryonic cells divide every two weeks during diapause. With developmental progression, we observed an increase in the number of expressed transcripts in the developing embryos. The pseudotime analysis of both embryos and LE showed grouping of the early blastocysts on one end and the elongated embryos on the other end of the trajectory. Between the two ends, the diapausing embryos were dispersed heterogeneously. Embryonic time-course analysis revealed 13,193 DET out of 29,575 transcripts. The DET grouped into 7 SOTA clusters. GO of the DET showed an enrichment of translational initiation and cell-cell adhesion in elongated embryos. The glycolytic pathway was high in early blastocysts and elongated embryos, but remained low during diapause. The LE was much less dynamic, with only 2,754 DET grouped into 2 clusters. During diapause, roe deer embryos divide at a slow pace and are transcriptionally active. Enriched pathways indicate cell proliferation and an increased energy demand after reactivation. Targeted transcript analyses will emphasise on the identification of diapause-related regulatory pathways and aim at identifying conserved mechanisms of cell cycle control.

## Macromolecule dynamics in the diapausing blastocyst in mouse

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Embryonic Diapause is a temporary arrest of blastocyst development which may last several days in mouse. The aim of this work was to verify whether changes in nucleic acids, proteins and lipids occur in blastocysts during the course of embryonic diapause, using techniques of mass spectroscopy. To this aim embryonic diapause was induced in pregnant mouse females by ovariectomy at 2.5 day post coitum (dpc) and daily injections of progesterone. Blastocysts were collected and analyzed every 2 days, from 4 to 12 dpc. Fourier Transform Infrared Spectroscopy (FT-IR) imaging analysis was chosen for the characterization of nucleic acids, proteins and lipids. Furthermore, lipid fingerprinting was characterized by Coherent anti-Stokes Raman spectroscopy (CARS) and by Matrix Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF/TOF). Our results show no difference in the viability of the embryos collected at different dpc. The blastocysts reached bigger size at 8 dpc, which was maintained till 12 dpc. FT-IR imaging analysis shows that spectra acquired for DNA/RNA content and protein structure differ between blastocysts collected at 4 to 12 dpc. There was a trend of reduction of lipids and lipid esters to protein ratio from 4 to 12 dpc. CARS analysis confirmed a reduction of lipid droplets with time. Lipids profile analysed by MALDI-TOF/TOF was performed for a major, phosphocholine (positive ion mode), and a minor, phosphoinositol (negative ion mode), component of biological membrane. Phosphocholine was also selected for its involvement in the implantation process: a peak of phosphocholine occur in the mouse uterus at the time of implantation. MALDI shows that specific ratio of phosphocholine (32:1), (34:1), (36:4) and (36:2), and phosphoinositol (18:0/16:1), (18:0/18:1), (18:0/20:4) and (18:0/22:4) are detectable for blastocyst from 4 to 12 dpc.

Our results indicate that the molecular composition of diapausing embryo differs in time analyzed suggesting that different cellular pathway are activated/inhibited and/or differently regulated at different time points. Relevantly, changes in the quantity of lipids indicate high demand of intracellular lipids during embryonic diapause.

## Autophagy regulates embryonic survival during delayed implantation in mice

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Autophagy is a major cellular catabolic pathway and is the only process known to degrade intact organelles. It is the primary intracellular mechanism for degrading and recycling long-lived proteins and organelles. Suboptimal extracellular environments (nutrient starvation, hypoxia, overcrowding) and intracellular stress (accumulation of damaged cytoplasmic components) induce autophagy. Diapausing blastocysts remain dormant for an extended period but resume implantation competence upon favorable conditions. The underlying mechanism by which extended longevity of dormant blastocysts is maintained is not clearly understood. We showed using the well-defined delayed implantation model in mice that dormant blastocysts exhibit heightened autophagic activation during delayed implantation. Activation of autophagy, the self-eating process within cells, seems to be an adaptive response to an unfavorable environment during prolonged survival in utero, as inhibiting autophagy reduces the survival rate of dormant blastocysts. In this model, prolonged dormancy led to reduced developmental competency of blastocysts and cellular damage with compromised pregnancy outcome. Estrogen supplementation to activate dormant blastocysts induces the accumulation of multivesicular bodies (MVBs) in the trophectoderm in vivo. Since autophagosomes are shown to fuse with MVBs and efficient autophagic degradation requires functional MVBs, the aspects of MVB formation in activated blastocysts were investigated. We reported that autophagic activation during dormancy is one precondition for MVB formation in activated blastocysts. MVB formation in activated blastocysts after dormancy may be a potential mechanism of clearing subcellular debris accumulated during prolonged autophagy.

## Gliding into diapause: early development in the roe deer (*Capreolus capreolus*)

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During mammalian diapause, the embryo is arrested at the blastocyst stage. In diapausing carnivores and marsupials, growth arrest of the blastocyst is coincident with a quiescent corpus luteum (CL). In the roe deer, progesterone is produced throughout diapause and the blastocyst exhibits very slow growth. Up to date, early roe deer embryos of known age have not been described. Therefore, it is not known at which stage and at which pace the roe deer embryo enters diapause. Cycle synchronization and superovulation was performed in captive roe deer prior to artificial insemination (AI) and natural breeding, respectively. Superovulation response was evaluated based on ultrasonographic detection of CL formation on the day of *in vivo* embryo flushing and on plasma progesterone (P4) analysis. In total, 33 superovulation treatments resulted in  $6.6 \pm 2.2$  (mean  $\pm$  SD) CL per doe on the day of embryo flushing. The number of CL and P4 concentrations on the day of embryo flushing significantly correlated ( $p=0.04$ ). On days 6 and 7 post mating, 37 multicellular embryos up to the morula stage were retrieved from 8 does. On day 12 and 13, 28 embryos, mainly at the blastocyst stage ( $n=20$ ), were collected from 7 does. Compared to other diapausing and non-diapausing species such as the related bovine, embryo development in the roe deer seems to be decelerated prior to the blastocyst stage. The roe deer might therefore be well suited as a model to investigate mechanisms regulating embryonic growth velocity with respect to cell cycle control.

## Diapause without arrest? - Characterization of cell proliferation in roe deer blastocysts

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The roe deer (*Capreolus capreolus*) was the first mammals in which diapause has been described. While there is a clear developmental arrest in some species, such as mice and rats, the roe deer blastocyst has been reported to show a very slow, yet continuous growth. Whether this growth is accompanied by developmental progression and whether it is uniform in the trophectoderm and the embryoblast, is not known to date. We collected 209 roe deer blastocysts during local huntings from September to December 2018, covering the period of diapause and reactivation, by uterine flushing, followed by fixation in paraformaldehyde. We used immunofluorescence and light-sheet microscopy to quantify the fraction of cells expressing the proliferation marker Ki67. As already described in 1902 [1], we observed considerable morphological changes, i.e. cavity formation and transition to a disk like shape in the embryoblast during diapause. Our preliminary data show that 5-15% of cells during diapause are proliferating. This fraction appears to be comparable in both embryoblast and trophectoderm. A first analysis points towards reactivation initiating in the embryoblast before starting in the trophectoderm. Taken together, our findings do not indicate that there is a complete developmental arrest in the roe deer during the whole period of diapause. Rather, in roe deer development slowly, but continuously progresses during diapause. We now aim at expanding the set of markers to further characterize the fraction of cells in G1 phase and mitosis. Studying the differences between the molecular mechanisms of decelerating versus arresting cell cycle progression will not only improve our understanding of cellular homeostasis in physiological and pathological contexts, but also may help to develop novel artificial reproduction technologies.

[1] Keibl F. 1902. Die Entwicklung des Rehes bis zur Anlage des Mesoblast. Arch Anat Phys Anat Abt. 1092:292-314

## POSTER PRESENTATIONS

### The roe deer oocyte transcriptome throughout the phases of embryonic arrest and reactivation

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Diapause is an embryonic developmental arrest reported in over 130 species. Nevertheless, its mechanisms are still not completely understood. In the roe deer (*Capreolus capreolus*), the only known ungulate that exhibits this phenomenon, embryonic arrest lasts for approximately five months, starting after the rut period in mid-July to early August and ending with embryo elongation and implantation in December/January. Little is known regarding oocyte characteristics during these periods. Here, we analyzed roe deer oocytes to understand the effects of embryonic developmental arrest (A) and reactivation (R) on the oocyte transcriptome. During regular huntings, immature oocytes were obtained by ovary slicing and classified according to morphological characteristics. Only oocytes with >2 layers of compact cumulus cells, and even cytoplasm from 30 hunted females were used for analyses. Immature oocytes were denuded and snap frozen. Additional oocytes were cultured in maturation medium for 20-24h. Matured oocytes with a present polar body were snap frozen. Two pools of 10 immature and mature oocytes for both embryonic developmental phases were included (at least four donors/pool). Oocyte pools were processed using the Smart-seq2 single cell protocol for full-length cDNA and library preparation. RNA-seq was performed on an Illumina HiSeq 4000 sequencer. The obtained Fastq files were submitted to sequence quality control and analyzed with a locally installed version of the Galaxy platform. Sequences were mapped against the roe deer transcriptome (unpublished data) and annotated against human, bovine and RefSeq transcripts. Differentially expressed genes (DEG, adj.pvalue <1%) were identified using ABSSeq. Comparisons included immature (IM) and mature (M) oocytes from the embryonic arrest (A) and reactivation (R) phases. Furthermore, to evaluate the effects of maturation on oocyte transcript abundance, DEG between IM and M oocytes for A and R were identified. Multidimensional scaling showed clustering according to oocyte types. Gene ontology (GO) terms for biological processes were assigned using ToppCluster tools. Differential transcriptomic regulation in roe deer oocytes was evident between the embryonic developmental arrest and reactivation phases. We found 68 and 48 DEG in immature and mature oocytes, respectively. Furthermore, maturation changed the oocyte expression profiles during embryonic arrest (1221 DEG) and reactivation periods (2534 DEG). GO revealed that most of the DEG are involved in cell cycle, RNA processing and transcription, mitochondria and mitochondria organization, metabolism and oxidative stress. These preliminary results suggest that oocyte transcriptome analysis might disclose novel mechanisms implicated in oocyte competence.

## Prostaglandins in the uterine fluid of the European roe deer (*Capreolus capreolus*)

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The European roe deer (*Capreolus capreolus*) exhibits delayed embryonic implantation during pregnancy, a phenomenon that is referred to as embryonic diapause. Rut takes place between late July to early August. The blastocyst enters the uterus presumably around the hatching stage. Its developmental pace is largely reduced, as embryonic cells divide approximately no more than every other week and the embryo reaches the size of about 40,000 cells only in late December. Thereafter, the embryo resumes growth, rapidly elongates and eventually implants in the endometrium of the uterus. The exact molecular mechanisms terminating diapause and leading to the resumption of embryonic growth have not been resolved yet. Prostaglandins (PG), which are local hormonal mediators derived from lipids potentially play a role therein. In humans, mice and domestic species, PG are involved in ovulation, luteolysis, pregnancy recognition and embryo implantation. The roe deer as seasonal, mono-ovulatory breeder does not need to inhibit luteolysis during the peri-implantation period as other ruminants. Thus, the role of embryonal PG regulating the continuously adapting maternal support necessary for embryo elongation and implantation may be elucidated in this model species. We have recently determined the PG secretion of the peri-implantation bovine embryo using a novel ultraperformance liquid chromatography coupled with mass spectrometry (UPLC-MS) based approach, showing that bovine embryos predominantly secrete PGE<sub>2</sub> and Δ12-PGD<sub>2</sub> until day 12 post insemination. Thereafter, PGI<sub>2</sub> (assessed by the determination of its stable metabolite 6keto-PGF<sub>1α</sub>) and PGF<sub>2α</sub> become predominant (day 14 post insemination). Similar to the bovine embryo, we hypothesize that the peri-implantation roe deer embryo secretes PG as local signalling molecules. We therefore aimed at determining PG in the uterine fluid of 80 pregnant female roe deer sampled from mid-September until end of December spanning the period of diapause and embryonic reactivation. Quantitative PG analysis was performed on a Waters nanoAcquity UPLC system equipped with a self-packed column (200 μm i.d. x 50 mm length) filled with HSS T3 C18 (1.8 μm particle size, Waters) coupled to a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer operating in Full Scan (AIF) mode. The approach includes 1-series, 2-series and 3-series PG and metabolites allowing for a PG profiling in roe deer uterine fluid. In a first subset of samples, we found an increase of PGF<sub>2α</sub>, 11β-PGF<sub>2α</sub>, 13,14-dihydro-15keto-PGF<sub>2α</sub> and 6keto-PGF<sub>1α</sub> with the developmental progression of the embryo. All further samples currently under analysis will confirm whether these preliminary findings are substantiated and verify if PG serve as local mediators of diapause in the European roe deer.

## Uterine fluid amino acids and acylcarnitines indicate metabolic activity in European roe deer (*Capreolus capreolus*) embryos during diapause

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Pre-implantation embryo development past the blastocyst stage depends on uterine secretions and well-orchestrated embryo-maternal interactions. With developmental progression, embryos have an increasing energy demand. In the roe deer, fertilisation and embryo implantation are decoupled by a 4-5 month period of embryonic diapause. Up to date, the mechanisms regulating diapause and reactivation of embryo development remain unknown in roe deer. Polyamines have been shown to play a role in embryo reactivation in mink. As arginine and ornithine are precursors for polyamine synthesis, amino acids can play a role in the reactivation of embryo development. The uterine fluid amino acids and acylcarnitines of 180 roe deer covering the period of embryonic diapause and reactivation were analysed to provide insight into the changing metabolic demands, and to shed light on the role of amino acids in the regulation of diapause. A total of 387 female roe deer was sampled between October – December 2015, September 2016 – January 2016, and September 2017 – January 2018. Five developmental stages including early (<833 cells per embryo), mid (833 – 1,666 cells per embryo), and late diapause (1,667 – 4,166 cells per embryo), pre-elongation (4,167 – 16,666 cells per embryo), and elongated (>16,667 cells per embryo) were defined. A targeted LC-MS/MS approach was used to quantify 30 amino acids in the uterine fluid of the selected 180 roe deer. The amino acids and acylcarnitines profiles were analysed by a between-group analysis, correlation analysis, and single analytes were plotted in R with a LOESS regression. The amino acids and acylcarnitines showed a developmental-stage specific abundance profile, most analytes correlated significantly, and most changes coincided with embryo elongation. Developmental progression was characterised by an increased abundance of valine, glutamine, glutamate, arginine, and ornithine. Glycine and alanine increased until elongation, but decreased after that, and the abundance of serine decreased from early diapause until elongation. The most abundant acylcarnitines at all stages included C0, C2, 2M-C3, C4, and C3. Major changes in acylcarnitine profiles occurred prior to elongation, indicating an increased energy demand around the time of reactivation of embryo development. These novel results provide evidence of metabolic activity in diapausing roe deer embryos. The increase in glutamine, arginine, and ornithine open doors for further research into conserved mechanisms of diapause.

## Evaluation of extracellular vesicles in the uterine fluid during embryonic diapause

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Extracellular vesicles (EVs) are a group of cell-derived membrane structures comprising exosomes (30-100 nm) and microvesicles (150-650 nm), which originate respectively from endosomal system or which are shed from the plasma membrane. They can contain small RNAs, mRNA and proteins that affect cells at distant sites. EVs have been identified in vivo in different biological fluids relevant in reproduction such as seminal, follicular and uterine fluid with a pivotal role in intercellular communication. Endometrial epithelium-derived EVs are involved in the communication between endometrium and blastocyst. Furthermore, blastocyst derived-EVs mediate the communication between inner cell mass and trophectoderm. EVs released from embryos and endometrium into the uterine fluid may influence the endometrial receptivity and implantation. During embryonic diapause, the endometrium is not receptive and the blastocyst is quiescent. We investigated the profile of EVs in the uterine fluid during embryonic diapause in mouse. To this aim, after natural mating the ovariectomy has been performed in mice at 2.5 day post coitum (dpc) to induce diapause and the uterine fluid has been collected at 6, 8, 10, 12 dpc. Non-diapausing control consisted of uterine fluid collected at 4 dpc. After isolation of EVs by series of centrifugations, sample concentration and size were analyzed by Nano Tracking Analysis (NTA). NTA showed an increase of the EVs number from 6 to 12 dpc vs. control. Qualitative evaluation was performed using Flow Cytometry and RAMAN spectroscopy. Flow cytometry using Annexin V (which label the phosphatidylserine in the EVs outer membrane leaflet) show an increase of Annexin positive events especially in the exosomes (30-100 nm) at 6, 10, 12 dpc vs. control. RAMAN analysis showed that the EVs are enriched of nucleic acids at time point 10 dpc, suggesting an activation of paracrine communication. In conclusion, these data demonstrated a communication between not receptive uterus and diapausing blastocysts. The limitation of this finding is the difficulty in distinguishing the origin of the EVs (endometrial epithelium or from the blastocyst).

## Behavioral screening of offspring obtained after embryonic diapause

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Embryonic diapause (ED) is a temporary arrest of embryo development and is characterized by delayed implantation in the uterus. ED occurs in blastocysts of several mammalian species, including the mouse (*Mus musculus*). Embryonic diapause studies are mainly focused on the analysis of the embryo itself, and no data about behavioral evaluation of the offspring obtained after ED are available. The aim of this study was to evaluate normalcy of offspring obtained after embryonic diapause using a battery of behavioral tests. Naturally mated animals were used in mouse model of ED. On 2.5 day post coitum females were ovariectomized to avoid estradiol surge indispensable for implantation in mouse. Blastocysts collected at different time points of ED were transferred to synchronized recipient females. Pups were allowed to be delivered spontaneously and weaned after 28 days. Each animal (3-4 months old) was subjected to three different tests. Motor coordination and synchrony was examined during normal walking in footprint test. The repetitive/persistent behavior was analyzed using marble burying test (the number of marbles buried serves as a proxy measurement for the extent of repetitive digging behavior). The light/dark transition test (LDT) was used to measure anxiety-like behavior and depressive-like behavior in offspring. Data collected from all three tests show similar behavior of offspring born following embryonic diapause.

## Profile of changes of the level of Treg lymphocytes in the blood and macrophages in the uterus of mice during embryonic diapause

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Regulatory T-cells (Treg) are immune cells that act to suppress immune response, thereby maintaining self-tolerance and homeostasis. Treg cells are involved in protection of allogenic conceptus against rejection by the mother immune system as well as preventing the development of hostile environment in uterus. Their role includes also the activation of other immune cells such as macrophages, which are phagocytic cells. It is known that uterine macrophages coordinate the remodelling of the uterus providing an appropriate environment for blastocyst implantation. Both Treg lymphocytes and macrophages are important part of the normal process of blastocyst implantation. During embryonic diapause (ED), the cross-talk between blastocyst and uterus is still poorly understood. The aim of present study was to evaluate the level of macrophages in the uterus and Treg lymphocytes in the peripheral blood during ED. For this purpose, female mice were mated and then, to avoid embryo implantation, ovariectomized at 2.5 day post coitum (dpc). In the subsequent days (i.e. 4-36 dpc), females were sacrificed and from those having diapausing embryos present in the uterus, horns of the uterus and peripheral blood from heart were collected. The peripheral blood was then analysed by flow cytometry for Treg lymphocytes, and the slides of uterine tissue were immunohistochemically stained for the presence of macrophages, which were then counted using a light microscope. The obtained results show periodic increases in the amount of Treg lymphocytes in peripheral blood as well as macrophages in the uterine endometrium. There was an increase in the amount of Treg lymphocytes in blood on days 6, 12 and 24 dpc, and similarly, an increase in the amount of macrophages in the uterine tissues on days 6 and 14 dpc (further dpc were not available at the time of analysis). The obtained data suggest a potential contribution of macrophages and Treg lymphocytes in maintaining the embryo diapause.

## Quantitative imaging of lipids in oocytes obtained from diapausing mammals

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Embryonic diapause (ED) is a period of temporary suspension of development at the blastocyst stage which occurs while waiting for the maternal implantation signal.

Minimal cell division which characterize ED, is possible likely due to the accumulation of energy reserves stored in oocytes in form of lipid droplets. ED has been observed in over 130 species of mammals, ranging from bears and badgers to mice. The aim of this study was to find out if the quantity of energy reserves in the oocytes is correlated with the duration of ED.

To do this, we applied a novel spectroscopic technique, chemically specific, label-free - Coherent Anti-Stokes Raman scattering (CARS) to estimate the amount of lipid droplets in oocytes collected from different diapausing mammalian species such as roe deer, European badger, American mink, bank vole and domestic mouse. The CARS imaging enabled to assess the number and mean size of lipid droplets, their spatial distribution as well as to quantify the total amount and content of lipids in the cell. The results of the analysis revealed the great variability in the oocytes' lipid content between examined species. We observe that the amount of lipid droplets is positively correlated with the ED duration in given species ( $p = 0.0464$ ). Data from our study proved an important relationship between lipids' content in the oocytes and ED length. These results confirm that the duration of the diapause in given species is related to the quantity of energy stored in form of lipid droplets in the oocyte.

## Post-natal growth and fertility of mouse offspring developed following embryonic diapause

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Embryonic Diapause (ED) is a temporary arrest of embryonic development in the uterus while waiting for the signal to implant. The aim of this work was to assess growth and fertility of mouse offspring obtained following ED. In order to induce embryonic diapause, females were mated with fertile males and then subjected to ovariectomy on 2.5-day post coitum (dpc) and daily injected with progesterone. Then, diapausing embryos were collected by uterine flushing every two days between 6.5 dpc and 24.5 dpc and transferred to pseudopregnant females ( $n \geq 4$  females/time point) for further development (ED group). Control group (CTR) consisted of 3.5 dpc embryos developed in non-ovarectomised females and transferred to pseudopregnant synchronized females, which were allowed to deliver spontaneously. Offspring was subjected to basic clinical evaluation, including onset of physical landmarks (fur and ear development, teeth eruption and opening of eyes), body weight, general welfare (e.g. body condition, hair coat condition) and fertility.

Diapausing embryos were able to develop to term until 18.5 dpc. No deliveries were obtained following transfer of diapausing embryos collected at 22.5 and 24.5 dpc. Sex ratio was shifted to an increased number of males in offspring developed following ED vs CTR (68% vs. 40%). There were no differences in growth, in terms of fur and ear development, teeth eruption and opening of eyes, and in body weight of pups developed following ED vs CTR. Furthermore, similar growth and health were observed offspring from ED and CTR in adulthood. Adult mice developed following ED were fertile (both males and females). In conclusion, our results show normal post-natal development and fertility of mouse offspring developed following embryonic diapause.

## Appendix C. Sponsors of the Symposium

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