Université de Montréal

Embryonic and Uterine Characteristics of Diapause

par
Evelyn Mercedes Llerena Vargas

Département de biomédecine vétérinaire
Faculté de médecine vétérinaire

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Présenté par :
Evelyn Mercedes Llerena Vargas

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Alan K. Goff, président-rapporteur
Bruce D. Murphy, directeur de recherche
Lawrence C. Smith, membre du jury

RÉSUMÉ

L’implantation retardée ou diapause embryonnaire décrit l'arrêt ou le retardement pendant l'embryogenèse. Chez le vison, la diapause est corrélée avec une sécrétion pituitaire insuffisante de la prolactine, ayant pour résultat la différentiation incomplète du corpus luteum et réduction de la progestérone. Des études antérieures suggèrent que le blastocyste de vison en diapause demeure dans un état de quiescence ou se développe lentement. Pour élucider ceci, la réplication de l'ADN a été étudiée. Les résultats démontrent synthèse de l’ADN et prolifération cellulaire dans les embryons au stade de morula, avant la diapause et dans les blastocystes après la réactivation. La réplication de l'ADN a été également détectée dans des blastocystes en diapause et en diapause prolongée. L'implantation est considérée comme une interaction bidirectionnelle entre le blastocyste et l'utérus. Il a été montré que les prostaglandines sont importantes pour la vascularisation de l’utérus au moment de l’implantation et peuvent réactiver l'utérus des visons après la diapause. La concentration protéinique et la localisation de la phospholipase citosolique A2 (CPLA2) et de la cyclooxygenase 2 (COX2) ont été étudiées dans l'utérus de vison. L’expression de la CPLA2 et COX2 étaient sur-régulées au moment de l'implantation. Il est connu que la prolactine active les corpus luteum des visons. L'idée de un lien entre la prolactine et la voie de signalisation des prostaglandines a été testée en mesurant les récepteurs de prolactine. Les résultats montrent une augmentation de l’expression des récepteurs de prolactine à l'implantation suggérant que la prolactine pourrait activer la voie de prostaglandine à l'utérus par son propre récepteur. La conclusion, les embryons pendant la diapause ne sont pas arrêtées complètement et les protéines liées à la voie de prostaglandine sont implique dans la réactivation de l'utérus.

Mots-clés : Diapause, implantation, réplication de l'ADN, CPLA2, COX2, PRL-R

ABSTRACT

Delayed implantation or diapause describes arrest or retardation during embryogenesis. In mink, diapause is related to insufficient pituitary prolactin secretion, resulting in incomplete differentiation of the corpus luteum with reduced progesterone concentration. The mink blastocyst at diapause was believed to be totally quiescent or expanding at a low rate. To explore this, DNA replication was studied. Results showed synthesis of DNA, and thus cell proliferation at the morulae stage before diapause and at the blastocyst following activation. DNA replication was detected not only at diapause but also at extended diapause. Furthermore, implantation is considered as a two-way interaction between the blastocyst and the uterus. It has been shown that prostaglandins are important for vascularization of the uterus and products of the prostaglandin pathway could reactivate the mink uterus following diapause. Protein concentration and localization was studied for cytosolic phospholipase A2 (CPLA2) and cyclooxygenase 2 (COX2) in mink uterus. Expression of CPLA2 and COX2 was up regulated at implantation. It is know that prolactin is the factor that activates the mink corpus luteum. The idea of a link between prolactin and prostaglandin pathway was investigated by quantifying the prolactin receptors in the uterus. Results showed an increase of prolactin receptors at implantation suggesting that prolactin could activate the prostaglandin pathway at the uterus through its own receptor. In conclusion, embryos during diapause are not completely arrested, and proteins related to the prostaglandin pathway are implicated in reactivation of the uterus.

Keywords : Diapause, implantation, DNA replication, CPLA2, COX2, PRL-R

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LIST OF ABBREVIATIONS

AA	Arachidonic Acid
AP2	AP2 transcription factor
BSA	Bovine Serum albumin
Ca^{+2}	Calcium ion $^{+2}$
cc	cubic centimetre
Cdk	Cyclin-dependet kinase
CEUA	Comité d'étique de l'utilization des Animaux
CL	Corpus luteum
COX	Cyclooxygenase
COX1	Cyclooxygenase 1
COX2	Cyxlooxygenase 2
CPLA2	Cytosolic phospholipase A2
CY3	Cyanine 3 dye
°C	Degree centigrades
DAB	3,3'-Diaminobenzidine tetrahydrochloride
DAPI	Diamino-2-phenylindole
DEDTC	Diethyldithiocarbamic acid
DNA	Desoxyribonucleic acid
e.g.	For example *(exampli gratia)*
EDTA	Ethylenediaminetetraacetic acid
Edu	5-ethynyl-2'-deoxyuridine
EP2	Prostaglandin E2 receptor 2
EP4	Prostaglandin E2 receptor 4
ER	Endoplasmic Reticulum
Flt1	Fms-like tyrosine kinase
G	Gauge
G0	G zero phase of the cell cycle
G0/G1	Arrest between G0 and G1 in the cell cycle
G1	Gap phase before synthesis

G1/S	Arrest in the cell cycle between G1 and S
G2	Gap phase before mitosis
G2/M	Arrest in the cell cycle between G2 and M
GnRH	Gonadotrophin Releasing Hormone
h	Hours
HCl	Hydrochloric acid
HRP	Horseradish peroxidase
ICM	Inner cell mass
IF	Immunofluorescence
IgG	Immunoglobulin G
IHC	Immunohistochemistry
iPLA2	Ca^{2+} -independent phospholipase
kDa	Kilo Daltons
KDR	Kinase domain region
µg	Micrograms
µl	Microliters
µm	Micrometers
M	Mitosis
MAPK	Mitogen activated protein kinases
MCF7	Michigan Cancer Foundation 7 cells
min	Minutes
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
ng	Nanograms
nm	Nanometer
%	Per cent
p.c.	Post coitum
PAF	Platelet-activating factor
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline with Tween

PGE2	Prostaglandin E2
PGH_2	Prostaglandin H_2
PKA	Protein kinase A
PRL	Prolactin
PRL-R	Prolactin receptor
PVDF	Polivinil difluoride
S	Phase of synthesis of DNA
SEM	Standard error of the mean
SP1	SP1 transcription factor
SPLA	Secretory phospholipase
Tris	Tris(hydroxymethyl)aminomethane
TTBS	Tris-buffered saline with Tween
V	Volts
VEGF	Vascular endothelial growth factor
ZP	Zona pellucida

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INTRODUCTION

Embryonic development begins when a spermatozoid fertilizes an oocyte released from the ovary, resulting in the formation of a zygote in the oviduct. The zygote undergoes mitotic cell division and reaches the morulae stage. Then, the morulae undergoes further mitotic divisions and cell differentiation, a process known as blastulation, to become a blastocyst. This blastocyst is surrounded by the zona pellucida (ZP), glycoprotein coat derived from the oocyte, and has two different cell populations: the trophoblast cells, which will give rise to the placenta and extraembryonic tissues, and the inner cell mass (ICM) that will give rise to the fetus. During its development, the embryo travels from the oviduct to the uterus where it will implant, after hatching from the ZP. In many carnivores the embryo implants through the ZP rather than hatching prior to attachment to the endometrium.

In most species of mammals, embryonic development described above proceeds without delay, and is therefore considered to be continuous. Examples of this continuous embryonic development include primates and most ruminants. However, the pattern of embryonic development from fertilization to implantation varies between species (Pike 1981). For instance, there is another type of embryonic development where implantation is postponed for a certain period of time during which the embryo is dormant (Dey, Lim et al. 2004). This embryonic development, a characteristic of my experimental model the mink *(Neovison vison*), is known as discontinuous embryonic development, also called delayed implantation or diapause. During the embryonic development the embryo should finish its differentiation for becoming ready for implantation. At the same time, the transforming uterus will reach the receptive state for embryo attachment. A two-way interaction for the implantation between the embryo and the uterus is necessary. For this reason, this project has two approaches during delay implantation in the mink. In the embryo approach, embryo

replication rate has been studied to elucidate the events associated with embryo dormancy during diapause. In the uterine approach, prostaglandin pathway has been investigated to determine its importance in the emergence from diapause. The objectives were to explore the protein concentration of cytosolic phospholipase A2 (CPLA2) and cyclooxygenase 2 (COX2) proteins, block the COX2 protein action, and to investigate the expression of prolactin receptors (PRL-R).

REVIEW OF LITERATURE

Delayed Implantation

The term delayed implantation or diapause describes any arrest or retardation of development during embryogenesis. Delayed implantation or diapause occurs in seven orders of mammals: Marsupials, Insectivora, Chiroptera, Edentata, Carnivora, Rodentia, and Artiodactyla (Mead 1993). There are two types of delayed implantation or diapause: facultative diapause and obligate diapause (Mead 1993).

Facultative Diapause

Facultative diapause occurs under conditions of metabolic and other stresses, the best known of which is lactation. Facultative diapause is restricted to species that exhibit a postpartum estrus few hours after birth while females are nourishing litters (Mead 1993). In female mice, estrogen, secreted by the stimulation of follicle-stimulating-hormone (FSH) which is controlled by the hypothalamic gonadotrophin releasing hormone (GnRH), is necessary for the implantation at day 4.5 post coitum (p.c.). The lack or insufficiency of estrogen secretion, is due to an inhibition of GnRH secretion (Coppings and McCann 1979), as a consequence of lactation and is related to the number of young suckled (Gidley-Baird 1981) results in facultative diapause in mice. Facultative diapause can also be induced in the laboratory by ovariectomy after mating and before the estrogen required for implantation is secreted, resulting in failure of implantation and initiating a state of dormancy of the mouse blastocyst. To maintain this dormancy in the mouse, progesterone treatment is necessary until the reactivation of the uterus and the reactivation of the embryo from arrest with an injection of β-estradiol (Schlafke and Enders 1975).

Obligate Diapause

Obligate diapause occurs in every gestation in my experimental model, the mink, a member of the Family Mustelidae. In the mustelids, three different reproductive patterns are exhibited, some species (ferret and least weasel) breed during spring and summer and do not exhibit diapause; others (mink and striped skunk) exhibit variable gestation periods with brief periods of diapause; and most mustelids (western spotted skunk, badger, marten, wolverine, etc.) always exhibit a prolonged diapause that lasts for several months, as much as 200 days in the spotted skunk (Mead 1981). An explanation for why obligate diapause occurs is that at high latitudes, where mustelids live, winter temperatures can be hostile for the survival of neonatal and young animals. As a consequence, these species mate when there are appropriate conditions for encountering mates (Lariviere and Ferguson 2003). For instance, my experimental model, the mink mates between the end of February and March. Pregnant female mustelids have a high demand of food until the delivery of newborns, explaining why embryo implantation is delayed until food is more available. Parturition is timed, so the young are born in spring (Mead 1989), allowing the maximum advantage of growth and to survival of offspring (Lariviere and Ferguson 2003) before their first winter. It is therefore believed that the evolution of diapause is related to adaptation because it allows mating and parturition to be uncoupled (Thom, Johnson et al. 2004) allowing the females and litters maximal survival benefit.

Embryonic Diapause and its Control

Obligate diapause is believed to result from insufficient pituitary secretion, resulting in incomplete differentiation of the corpus luteum (CL) (Mead 1993) which retains its mitotic potential during the period of diapause (Douglas, Houde et al. 1998). However, in order to maintain blastocyst viability during diapause the CL secretes small concentration of progesterone (Mead 1993). It is known in the mink that the change of

photoperiod related to the vernal equinox (21st of March in the Northern Hemisphere) begins the reactivation of the embryo and uterus (Figure 1). It was also shown in hypophysectomized mink treated with prolactin (PRL) that PRL activates the prolactin receptor (PRL-R) in the ovary (Douglas, Houde et al. 1998) activating the CL (Murphy, Concannon et al. 1981). As a consequence of the rejuvenation, the CL increases the progesterone secretion permitting embryo implantation and ending diapause (Murphy, Concannon et al. 1981). In conclusion, prolactin under the photoperiod influence is considered as the luteotrophic factor essential for embryo implantation in the mink.

The Embryo during Diapause

The blastocyst during diapause is either believed to be totally quiescent, with no mitotic activity, or to be expanding at a very low rate (Renfree and Callaby 1981; Surani and Fishel 1981). Blastocysts from mice and rats enter into quiescence between 100 and 200 cells (Surani and Fishel 1981) and after hatching from the ZP. Diapaused blastocysts from carnivores, as the mink, consist of 200 - 400 cells with an ICM made from 28 to 164 cells and the ZP is supplemented with layers of glycoprotein acquired during the passage of the embryo from the oviduct to the uterus (Enders 1952).

During diapause in the mink, there is no published evidence of trophoblast cell number increase. In the western spotted skunk, little protein synthesis occurs in the reactivated blastocyst suggesting reduced metabolic activity during diapause (Mead 1989). Moreover, the embryo diameter of the mink embryo remains around 200 μm at diapause, increasing after reactivation to 1.2 mm (Martinet, Allais et al. 1981).

Cell Cycle

As noted in various reviews, the cell cycle is a series of processes by which the cell divides to give rise to two daughter cells (Schafer 1998; Johnson and Walker 1999; Vermeulen, Van Bockstaele et al. 2003). The eukaryotic

cell cycle comprises four phases; G1 gap phase where the cells prepare for the desoxyribonucleic acid (DNA) replication, S phase for DNA synthesis, G2 gap phase where the cells prepare for mitosis, and M or mitosis phase. The first three phases are considered part of the interphase. Furthermore, in the cell cycle, there are two different checkpoints, the first between the G1/S phases and the second between G2/M phases. An arrest in the different checkpoints is consequence of DNA damage, of activation or inactivation of proteins related to the cell cycle, or activation of inhibitors.

Important proteins for the cell cycle progression are the cyclins and cyclin-dependent kinases (cdk). It is believed that the cdks, activated by the binding to cyclins, provide the first mechanism for cell cycle regulation. For instance, Cyclin D associated to cdk2 or cdk4 is important for the entry into G1. Cyclin E associated to cdk2 helps the transition from G1 to S phase. Cyclin A associated to cdk2 and then cdk1 pushes the cell cycle to G1 phase. Finally, Cyclin B coupled to cdk1 drive the cell cycle to M phase. The second important proteins for the cell cycle are the inhibitors. The first example of inhibitors is the INK4 family (p16, p15, p18, and p19) and the other example of inhibitors is the cip/kip family of proteins (p21, p27, p57) that arrest the cell cycle at G1 (Vermeulen, Van Bockstaele et al. 2003).

The cell cycle stage at which mammalian embryos arrest during diapause has been hypothesized at the G1/S phase (Lopes, Desmarais et al. 2004). In dormant mouse embryos there is an up-regulation of $p21^{cip/WAF1}$; a down-regulation of Cyclin D1, Cyclin E1, and Cyclin E2 genes involved in the progression of the cell cycle; and down-regulation of Cdc6, Cdc45, Orc1, and Mcm5 genes involved in DNA replication (Hamatani, Daikoku et al. 2004) supporting the idea of G1/S arrest. In previous studies in the mink, diapause embryos cultured with 5-bromo-2-deoxyuridine did not display uptake, supporting the idea of arrest between G0/G1 (Lopes, Desmarais et al. 2004).

Embryo at Reactivation

Embryo reactivation after diapause in mice is considered a two step mechanism beginning with an increase in metabolic activity and finishing with an increase in outgrowth capacity (Weitlauf and Kiessling 1981). Cytological and metabolic changes within mustelids with prolonged delayed implantation blastocysts can be detected when they are undergoing renewed development, including an increase in polyribosomes and cell number in the trophoblast and inner cell mass (Mead 1989). Another change in mink embryos is in embryo size, apparently due to increased fluid uptake and protein and DNA synthesis within 72 h after reactivation (Desmarais, Lopes et al. 2007).

Objective I:

It is not well known if during diapause, there is an arrest in the cell cycle or not. As a consequence, the possibility of proliferation in the cell cycle in mink embryos during diapause is the basis of the embryo approach. For this reason, it is necessary to establish weather DNA replication occurs in three stages of diapause, the stage of entry into diapause, the stage of diapause, and the stage of reactivation.

The Uterus in Diapause

When the embryo reaches the uterus in the case of a continuous developmental trajectory, the process of implantation begins as in the mouse where the blastocyst arrives in the uterus on Day 3.5 post coitum (p.c.) and implantation begins on Day 4. However, this implantation process depends not only on the embryo, but also is considered as a two way interaction between the blastocyst and the uterus (Dey, Lim et al. 2004). For the uterus, the timing for implantation, known as “window of implantation”, begins when the uterus will allow the embryo attachment, known as the receptive state. This receptive state of the uterus results from a coordination of the endocrine, cellular, and molecular events via paracrine, autocrine, and/or

juxtacrine signals (Dey, Lim et al. 2004). These factors allow attachment of the trophoblast cells and cause the stroma cells to differentiate.

Hypotheses related to the Uterus during Diapause

There are two contradictory hypotheses surrounding the receptivity of uterus during diapause. Either the uterus secretes a factor that inhibits metabolic activity in the embryos; or the uterus fails to secrete a stimulatory factor (Weitlauf and Kiessling 1981). In the mink uterus, there is evidence suggesting that during diapause, it is the uterus that does not support implantation. The first evidence was an embryo transplant experiment where blastocysts from the ferret (a non-diapause species) were arrested in development when transferred to the mink uterus, while mink blastocyst reinitiated embryogenesis in the ferret uterus (Chang 1968). The second source was the observation of hatching of mink embryos from its ZP and trophoblast outgrowth of mink embryos co-cultured with immortalized mink uterine epithelial and stromal cells (Moreau, Arslan et al. 1995).

The earliest sign of blastocyst implantation in rodents is an increase in endometrial vascular permeability in adjacent areas to the blastocyst implantation and this is followed, in species with invasive implantation, by decidualization (Kennedy, Gillio-Meina et al. 2007). However, the mink does not present decidualization but angiogenic factors are necessary for the endotheliochorial placenta development that will support fetal development (Lopes, Desmarais et al. 2006).

Implication of Prostaglandin Pathway in Diapause

In the implantation process, prostaglandins are related to the increase of vascular permeability during implantation. Prostaglandins are implicated in ovulation and implantation due to their vasoactive, mitogenic and differentiating properties (Lim and Dey 1997). The prostaglandin pathway begins with the metabolism of arachidonic acid (AA) by means of enzymatic activity of a phospholipase (Gijon and Leslie 1999).

The mammalian phospholipase superfamily consists of four major subfamilies that include cytosolic (CPLA2α), secretory (SPLA), Ca^{2+} - independent (iPLA), and platelet-activating factor (PAF) acetylhydrolase. CPLA2 and SPLA2 generate fatty acids as AA, while $iPLA_2$ and PAF acetylhydrolase remodel the cell membrane and attenuate the PAF activity, respectively (Song, Lim et al. 2002). The lack of similarity between CPLA2 and other family members, suggests a unique role for CPLA2. CPLA2 is activated by increase in intracellular Ca^{+2} or phosphorylation by mitogen activated protein kinases (MAPK). Once activated, CPLA2 undergoes a translocation from the cytosol to the perinuclear and endoplasmic reticular (ER) membranes, the sites of cyclooxygenase (COX) enzymes (Gijon and Leslie 1999). It is believed that CPLA2 is attached around the perinuclear membrane by intermediate filaments like vimentin (Nakatani, Tanioka et al. 2000). The function of CPLA2 is to hydrolyse the ester bonds of fatty acids at the *sn-2* position of phospholipids, producing free fatty acids and lysophospholipids necessary for the prostaglandin production from AA (Song, Lim et al. 2002).

The implication of CPLA2 in implantation was shown with the use of knockout mice. For instance, it was shown that implantation is temporarily deferred in these mice by a shift of the normal window of implantation (Song, Lim et al. 2002), suggesting that the lack of this protein can induce a delay in implantation. Furthermore, CPLA2 mRNA was co-localized with the cyclooxygenase1 (COX1) mRNA, and cyclooxygenase2 (COX2) mRNA, in the preterm luminal epithelium and term uterine stroma cells respectively (Brown, Morrow et al. 2009).

The next proteins in the prostaglandin pathway are the two COX proteins with the function of converting AA to prostaglandin H_2 (PGH_2). It is known that COX1 is constitutively expressed, while COX2 is inducible (Dey, Lim et al. 2004). Implantation was compromised in mice by using DuP697, a selective inhibitor of COX2, suggesting that this protein is important for the implantation process (Lim, Paria et al. 1997). In the case of the mink, it has

been shown that COX2 mRNA was up-regulated in the uterus at Day 3 post-implantation and down-regulated from Day 3 post-implantation to Day 10. Moreover, the COX2 protein was localized in the mink uterine epithelium at Day 2 post-implantation and, somewhat later, in the stroma and uterine glands (Song 1998).

From $PGH_{2;}$ prostacyclins, thromboxanes, and the prostaglandins (E, F,and G) are formed by different pathways. In the case of the mink, it has been also shown that prostaglandin E_2 (PGE_2) stimulates vascular endothelial growth factor (VEGF) through a protein kinase A (PKA) dependent pathway and use EP2 and EP4 receptors for that function and also uses two regions of the VEGF promoter, AP2 and SP1 (Lopes, Desmarais et al. 2006). Moreover, it is known that VEGF is an important regulator of angiogenesis (Ferrara and Davis-Smyth 1997). In the case of the mink, it has been shown that three different VEGF isoforms (VEGF 120, VEGF 164, and VEGF 188) and VEGF receptors kinase domain region (KDR) and fms-like tyrosine kinase (Flt1) are up-regulated at late reactivation and implantation site by metabolites of the prostaglandin pathway (Lopes, Desmarais et al. 2003).

Objective II:

The uterine approach of my thesis is based in the idea that the prostaglandin pathway could be downregulated during diapause and could be reactivated during reactivation of the uterus following the exit from diapause in order to induce the uterus to become receptive for embryo implantation. To address this idea, as a first objective the protein concentration and protein localization of CPLA2 and COX2 proteins in mink uterus was investigated. As a second objective, COX2 action was blocked by Meloxicam in order to determine the effects on COX2 protein expression. As a third objective, prolactin receptors were investigated at the uterine level.

MATERIALS AND METHODS:

Animals and Sample Collection:

All animal experimentation was approved by "Le Comité d'étique de l'utilization des Animaux" (CEUA) de la Université de Montreal, under a Project Number of 11-Rech-1008. Pastel mink females from the farm of Mr. A. Richard (Saint Damase, QC, Canada) were mated twice to male mink, 7 days apart, according to standard farm procedures. For the uterine approach, female minks were euthanized at different time points over several breeding seasons. Females from the "diapause" group were euthanized 7 days after second mating. The remaining females were synchronized by using ovine pituitary prolactin (Sigma Aldrich, St. Louis, USA) diluted with autoclaved-mineral oil (Sigma Aldrich) in sterile single-use 25 G 5/8 (Becton Dickinson, Franklin Lakes, USA) needle and 3 ml (Becton Dickinson) syringe under sterile conditions for 3 days, 5 days, 9 days, 11 days, 13 days, and 15 days after the 21st of March. To examine the effects of inhibition of the COX2 action a Meloxicam experiment with a total of 15 females from the most recent season (2011) was performed. Females were separated in 3 groups (figure 12): 5 females from diapause and 10 females with 7 days of synchronization with ovine pituitary prolactin. From the last group of 10 females, 5 were injected subcutaneously with Meloxicam and the others left as a control group. For the embryo approach, female mink from the most recent season (2011) were separated into 4 different groups (figure 2). Animals in the first group, entry to diapause, were euthanized 5 days after second mating. The second group, diapause group, was euthanized 10 days after second mating. In the third group, group of long diapause, females were implanted with melatonin implants (kindly provided by Mr. Terry B. Cairns, Wildlife Pharmaceuticals) from the 20th of March until the 15th of April and euthanized the 15th of April. In the fourth group, reactivated group, females were injected daily with ovine prolactin for 7 days and

euthanized on the seventh day. For euthanasia, intramuscular anesthesia, mixture of ketamine and acepromazine in a sterile 1cc/ml (Becton Dickinson) syringes with 26 G x 5/8" needle (Terumo, Elkton, USA), was used. Blood samples were taken by cardiac puncture under anesthesia. Finally pentobarbital in a sterile 1 cc/ml (Becton Dickinson) syringes with 25 G x 5/8" needle (Terumo) was used to complete the euthanasia. Uterine samples were removed by midventral laparotomy and transported from the farm to the laboratory in 50 ml (Sarstedt, Saint Leonard, Canada) tubes with phosphate buffered saline (PBS) at 37 °C. Under sterile conditions, fat tissue was removed from uteri that were then flushed for embryo collection, and snap frozen in 1.5 ml (Progene, Saint Laurent, Canada) tubes using liquid nitrogen. Half of the uteri were kept at -80 °C until protein extraction. The remaining half uterus was fixed in 4 % paraformaldehyde (Sigma Aldrich) for immunofluorescent (IF) or immunohistochemical (IHC) analysis.

Mink Embryo Collection:

After separating ovaries and oviducts from uterine horns, the uterine horns were flushed with Medium 199 Hepes (Invitrogen, Carlsbad, USA), 10 % Hyclone Fetal Bovine Serum (Thermo Scientific, Freemont, USA), and penicillin-streptomycin (Invitrogen) for embryo collection. The liquid was collected on petri dishes (Nunc, Roskilde, Belgium). The embryo diameter was measured and used as a marker for separate embryos in morulae, blastocyst during diapause, and reactivated blastocyst by using the MS5 Stereo Microscope (Leica, Heerbrug, Germany). Embryos were cultured with M16 medium (Sigma Aldrich) supplemented with 1 mM sodium pyruvate (Invitrogen), L-glutamine (Invitrogen), penicillin-streptomycin (Invitrogen), and Click-iT EdU Imaging Kit (Invitrogen, Oregon, USA) following the manufacturer's instruction in a Multidish 4 well plates (Nunc) for 12 hours. Photomicrographs were taken using the ASLMD fluorescence

microscope (Leica) and DC500 camera (Leica). Verification of cell counts was achieved by using confocal Microscopy (Olympus FV1000, Leica).

Mouse Embryo Collection:

Adult C57B/6:129 wild type mice females from 3 to 5 months of age were maintained in controlled environment with a 14 h light/ 10 h dark cycle. They were mated with fertile males of the same strain to induce pregnancy (Day 1 = the day of vaginal plug). On the afternoon of Day 3 between 15:00 and 17:00 or in the morning of Day 4 between 09:00 and 11:00, females were bilaterally ovariectomized and received progesterone implants or were injected with progesterone (2 mg/0.1 ml, Sigma Aldrich) beginning the day after the surgery until euthanasia. Females were divided into groups designated as follows: Entry into Diapause (euthanized at Day 4.5 post-coitum (p.c.)), Diapause (euthanized at Day 7 p.c.), Long Diapause (euthanized at Day 11 p.c.), and Reactivation (females were injected with 25 ng/0.1 ml of estradiol, Sigma Aldrich) at Day 8 p.c., and euthanized 12 h later (figure 6). Females were euthanized by cardiac puncture under isofluorane anesthesia. The uterine tract was removed by midventral laparotomy and isolated in PBS at 37 °C and then horns were flushed with M2 medium (Sigma Aldrich) and penicillin-streptomycin (Invitrogen) on a petri dish (Nunc) using the MS5 Stereo Microscope (Leica). Embryos were cultured with the same medium as the mink embryos. Photomicrographs were taken as described for mink embryos.

Click-it EdU Labeling:

A total of 73 mink embryos and 9 mice embryos were cultured for 12 hours in culture medium plus 5-ethynyl-2'-deoxyuridine (EdU, Invitrogen). EdU, a nucleoside analog of thymidine, is used because it incorporates into DNA during active DNA synthesis, serving as a marker for the S-phase of the cell cycle. After 12 hours, the embryos were fixed with 3.7 % formaldehyde and permeabilized 0.5 % Triton X-100. After washing steps, embryos were stained with Alexa Fluor 594, dying the replicating cells in red. In the next

step, embryos were cultured with Hoechst 33342 (Invitrogen) dye for the nuclei. Red nuclei representing the replicating cells were recognized between 594 and 615 nm, and were counted. Blue nuclei recognized in a window between 350 and 461 nm, representing the total number of cells, were designated as 100 % and counted. The percentage of replicating cells was calculated by determining Hoechst cells as 100% and Alexa-Edu positive for replications as a percentage. Mean ratios were presented by calculating replicating nuclei (Alexa-Edu cells) over total numer of nuclei (Hoechst positive cells) and were presented in tables.

Protein Extraction:

Extraction buffer for protein isolation was prepared with a mix of sonication buffer (20 mM Tris-HCl pH 8.0, 0.05 mM EDTA, home-made), 0.1 mM diethyldithiocarbamic acid (DEDTC, home-made), 46.78 µg of n-OCTYL β-D-GLUCOpyranoside (Sigma Aldrich), PhosphoStop (Roche, Indianapolis, USA) and Complete Mini (Roche). To tubes of 1.5 ml was added 300 µl of extraction buffer and a pestle was used in order to disrupt 3 mm of uterine tissue at 4 °C. Samples in the tubes were centrifuged for 10 min at 4 °C in microcentrifuge (Fisher Scientific, Fair Lawn, USA). Protein extraction recovered from the pellet was stored at -80 °C until Bradford Assay.

Bradford Assay:

Bradford Assay was conducted in Microtest 96 wells (Sarstedt Inc.). A standard curve was employed with 10 µl of BSA at different concentrations (0 µg, 0.5 µg, 1 µg, 2 µg, 4 µg, 5 µg, 7 µg and 10 µg). The standard of 4 µg BSA was used as a positive control. An aliquot of 1 µl of each sample was diluted in 9 µl of distilled water. Finally 200 µl of BIO RAD protein Assay (BioRad, Hercules, USA) was added to each well. Protein quantity was determined by spectrophotometry, employing a Fusion Universal Microplate Analyzer (Perkin Elmer, Boston, USA).

Western Blot for CPLA2, COX2 and Prolactin Receptor:

Uterine samples from diapause, Day 3 of reactivation, Day 5 of reactivation, Day 9 of reactivation, Day 11 of reactivation, Day 13 of reactivation. Samples from 3 different individuals were tested per group. Total amount of protein (from 15 to 30 μg) was fractioned on 8 % polyacrylamide-biscrilamide (Fisher Scientific) gel for 2 h at 110 V. Gels were electroblotted to Amersham Hybond-P hydrophobic polyvinyl difluoride (PVDF) membranes (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions over-night at 4 °C. Membranes were probed with three different antibodies overnight at 4 °C, 1:200 of a rabbit polyclonal CPLA2 antibody (Sc-438, Santa Cruz Biotechnology), 1:1000 of COX2 Monoclonal antibody-Mouse clone CX229 (160112, Cayman), 1:500 of Mouse Monoclonal Prolactin Receptor antibody [U5] (2772, ab Cam), and Mouse monoclonal IgG β-actin HRP (Sc-47778, Santa Cruz Biotecnology) 1:100 000 as a control protein. After washing with Tris-buffered saline with Tween (TTBS), membranes were incubated 1 h with anti-rabbit peroxidase conjugated IgG (Cat.#111-035-003, Jackson Immuno Research Laboratories Inc, West Gove, USA) for CPLA2 Western Blot; with Rabbit anti-Mouse IgG HRP Conjugated (Cat.# 402335, CalbioChem, USA) for COX2 and Prolactin Receptor. Then, membranes were incubated for 5 min with Immobilon Western Chemiluminiescent HRP Substrate (Millipore, Billerica, USA) in the dark room. Films were developed using Amersham Hyperfilm ECL (GE Healthcare) film and Ecomax X Ray Film Processor (Protect GmbM & Co, Oberstenfeld, Germany). The Amersham Full-Range Rainbow Molecular Weight Markers (GE Healthcare) was used to estimate the protein band. Finally films were scanned with PSC2175 all-in one Hewlett Packard printer-scanner-copier. Band pixels were calculated using ImageJ software.

Statistical Analysis:

For western blot, the ratio between the protein and the control protein was used to compare the quantity of each sample. The Shapiro Wilk test was employed to test for normality of sample population. Western blot results were analyzed by ANOVA with JMP software (SAS Institute, Cary, NC) and group comparisons between means were made by the Tukey test. Statistical differences were recognized when $p < 0.05$. Embryos diameter and percentage of replicating cells were analyzed by a linear model for heterogeneous variances followed by Tukey's post-doc with a $p < 0.0001$ and $p = 0.006$ respectively. All results were expressed in mean ± SEM.

cPLA2 Immunofluorescence:

All tissue blocks were sliced in 5 μm tissue sections using a rotary microtome Spencer AO Lab-Tek Microtome (American Optical Company, New York, US). Tissues slides were dewaxed twice in toluene for 5 min, and rehydrated in ethanol 100 %, 90 % and 70 %. Tissue slides were incubated in sodium citrate buffer at 95 °C for 5 min for antigen recovery. After the sodium citrate buffer cooled at room temperature (RT), tissue slides were rinsed with PBS with Tween 20 (PBST) 5 min 3 times. Tissue slides were blocked with bovine serum albumin (BSA) 5 % for 2 h and 30 min in a humid chamber at RT. Tissue sections were incubated with primary antibody of rabbit-polyclonal CPLA2 (Sc-438, Santa Cruz Biotechnology) 1:100 in BSA overnight at 4 °C. After being washed with PBST three times 5 min, tissue sections were incubated with secondary antibody CY3–conjugated goat anti-rabbit IgG (Jackson Immuno Research) 1:400 for 1 h at RT. The nuclei were stained with diamino-2-phenylindole (DAPI) 1:1000 (Sigma). Finally slides were sealed with a drop of mounting Permafluor (Thermo Scientific) and a coverslip. Images were captured at 200x magnification, using ASLMD fluorescence microscope (Leica) and DC500 camera (Leica).

COX2 Immunohistochemistry:

Tissue slides were dewaxed and rehydrated as above. Antigen recovery was achieved by incubation of tissue slides with 0.075 % trypsin 20 min at 37 °C. Peroxidase activity was saturated with an incubation with 0.1 % H_2O_2 (Fisher Scientific, Fair Lawn, USA) for 30 min. Tissue slides were blocked with bovine serum albumin (BSA) 5 % for 2 h and 30 min in a humid chamber at RT. Tissue slides were saturated for Avidin Biotin (Vector Laboratories, Burlingame, USA) for 15 min of each and a washing step in between. Slides were incubated with primary antibody (Sc 1746, Santa Cruz) 1:50 overnight at 4 °C. After being washed with PBST 5 min three times, tissue slides were incubated with secondary antibody biotinylated rabbit anti-goat IgG (Vector Laboratories) 1:200 for 1 h at RT. Tissue slides were exposed to 3,3'-Diaminobenzidine tetrahydrochloride (DAB - Sigma Aldrich) for 1 min. Nuclei from the tissue slides were colored with hematoxilin (Fisher Scientific) for 1 min. Tissue slides were dehydrated through a series of alcohols and xylene. Tissue slides were sealed with a drop of Permount (Fisher Scientific) and a cover slip. Images were captured at 200x magnification, using ASLMD fluorescence microscope (Leica) and DC500 camera (Leica).

RESULTS

Embryo Studies:

Embryo Diameter

Mink embryos at morulae stage were recovered from female mink groups at entry to diapause and diapause. Mink embryo diameters from 85 morulae, 20 blastocysts at entry to diapause, 28 blastocysts from diapause, 74 blastocysts from reactivation, and 5 blastocysts from long diapause were measured (Figure 3). The mean of the embryo diameter was significant different among morulae and blastocysts from diapause. Furthermore, blastocysts at reactivation (370.6 ± 18.8 μm) and in long diapause (695.0 ± 90.5 μm) were significant larger than those taken at other time points ($p < 0.0001$).

Replication Rate and Ratios

A total of 28 mink morulae, 3 mink blastocysts from the entry to diapause, 18 mink blastocysts from diapause, 21 mink blastocysts from reactivation, and 3 blastocysts from long diapause were cultured for replication rate experiment (figure 4). Mink morulae embryos (71.7 ± 2.8 %) and mink blastocyst at reactivation (75.3 ± 2.8 %) had the highest replication rate (figure 5). Furthermore, replication rate from mink blastocysts at diapause (50.3 ± 4.3 %) and at long diapause (23.7 ± 7.7 %) were the lowest. Blastocyst from early reactivation group had replication rates in between the groups with highest replication rate and the groups with lowest replication rate.

Considering the replication ratios, it was observed the same amount of cells in mink blastocyst at the entry to diapause and at diapause, but it got our attention that during long diapause the embryo showed a higher number of total cells compared to the other groups.

Time Points in Mink Embryos	Replicating Nuclei	Total Number of Nuclei	Ratios
Morulae	11 ± 4.41	16 ± 5.61	11/16
Entry to diapause	55 ± 13.24	104 ± 16.82	55/104
Diapause	46 ± 5.40	97 ± 6.87	46/97
Long diapause	62 ± 13.24	267 ± 16.82	62/267
Reactivation	125 ± 5.13	166 ± 6.51	125/166

Table 1: Mink Embryo Ratios

In the case of the mouse embryos, 7 morulae at Day 4.5, considered being at entry to diapause, and 2 blastocysts, at diapause, were collected. Replication rate from morulae (67.3 ± 5.5%) was significantly different from the blastocyst at diapause at $p < 0.05$ (figure 7). Paying attention to the replication ratios, it was observed that mouse embryos during diapause had a higher number of cells than the embryo at entry to diapause.

Time Points in Mouse Embryos	Replicating Nuclei	Total Number of Nuclei	Ratios
Entry to diapause (day 4.5)	10 ± 2.69	14 ± 1.58	10/14
Diapause (day 7)	17 ± 4.26	58 ± 2.51	17/58

Table 2: Mouse Embryo Ratios

Uterine Studies:

Production of Cytosolic Phospholipase A2 (CPLA2) in Mink Uterus Samples:

Western Blot analyses were performed using lysates of mink uterus samples at different time points. A band between 85 kDa and 114 kDa was expected. In the case of the mink samples, a band was detected between the 76 kDa and 102 kDa markers around 90 kDa. Protein extracted from Michigan Cancer Foundation-7 human breast cancer cells (MCF7) was used as positive control for CPLA2, and the band for those human cells had less migration that for the mink uterus samples. The concentration of CPLA2 at diapause and late preimplantation was significantly greater than at early

preimplantation. The concentration of the protein at diapause and late preimplantation did not differ. CPLA2 was had the highest concentration at implantation with a $p < 0.05$ (Figure 8).

Localization of CPLA2 in Mink Uterus:

To localize CPLA2 protein, uterine tissues from mink were sectioned and immunofluorescence analyses were performed (Figure 9). The results show that CPLA2 protein was present in luminal epithelium and in the epithelial cells from the glands. We saw CPLA2 protein localization at diapause and early preimplantation in both luminal epithelium and epithelial cells from the glands. However, in implantation site and in regions between implantation site CPLA2 protein was localized only in the epithelial cells from the glands.

Abundance of Cyclooxygenase2 (COX2) Protein in Mink Uterine Samples:

Western blot analyses were performed using lysates of mink uterus samples from different time points during gestation. A band around 72 kDa was expected. In the mink uterus samples, three bands were detected. A lysate of proteins extracted from bovine granulosa cells cultured with LH was used as positive control (kindly provided by Gustavo Zamberlam). In this positive control, the same three bands were present at the same approximate molecular weight. However, considering that COX1 migrates as two bands, one around 69 kDa and another around 72 kDa (O'Neill, Mancini et al. 1994), the highest band around 74 kDa was selected as the COX2 band. The abundance of the protein form of COX2 at diapause, early preimplantation and late preimplantation did not differ among the stages of study (Figure 10). COX2 protein concentration was significantly increased in implantation in comparison with the other 3 groups $p < 0.05$.

Localization of COX2 in Mink Uterus:

To localize COX2 protein, mink uterine tissues were sectioned and immunohistochemistry was performed. The results showed that COX protein was present at the luminal epithelium and epithelial cells from the glands (Figure 11). The results showed elevated expression of COX2 protein at implantation in the luminal epithelium and some epithelial cells from the glands.

Effect of Meloxicam on COX2 Expression in Mink Uterus:

Western blot was performed to explore the difference in COX2 concentration in three different groups (diapause, early preimplantation, and inhibition at early preimplantation). The results showed that COX2 protein concentration from diapause, early preimplantation, and inhibition at early preimplantation did not differ (Figure 13).

Effect of Meloxicam in the Localization of COX2 in Mink Uterus

Immunohistochemistry was performed to establish the difference in COX2 protein localization in three different groups (diapause, early preimplantation, and inhibition at early preimplantation). The results were not credible because of background seen in the pictures at different stages probably due the incubation with DAB (figure 14). A mink implantation sample was used as a positive control (Figure 11d). There, it is easy to see the localization of COX2 at the luminal epithelium and the glands in comparison with the rest of the tissue.

Expression of Prolactin Receptors (PRL-R) in Mink Uterus:

Western blot was performed in mink uterine samples from different time points in early gestation. Three bands were expected: a short isoform at 40 kDa, a medium isoform at 60 kDa, and a long form at 85 kDa. MCF7 protein extract was used as positive control. In the mink uterus, two isoforms of PRL-R were seen migrating at approximately 40 and 60 kDa.

However, since the 60 kDa isoform was not presented in all the samples, the 40 kDa isoform was quantified. The results showed that there is an increase of PRL-R from diapause through implantation. Diapause and early preimplantation did not display a difference in PRL-R concentration. Implantation had the highest concentration differing significantly from diapause, early preimplantation and late preimplantation ($p < 0.05$), while late preimplantation had PRL-R concentration in between the implantation and the lowest concentration at diapause and early implantation (Figure 15).

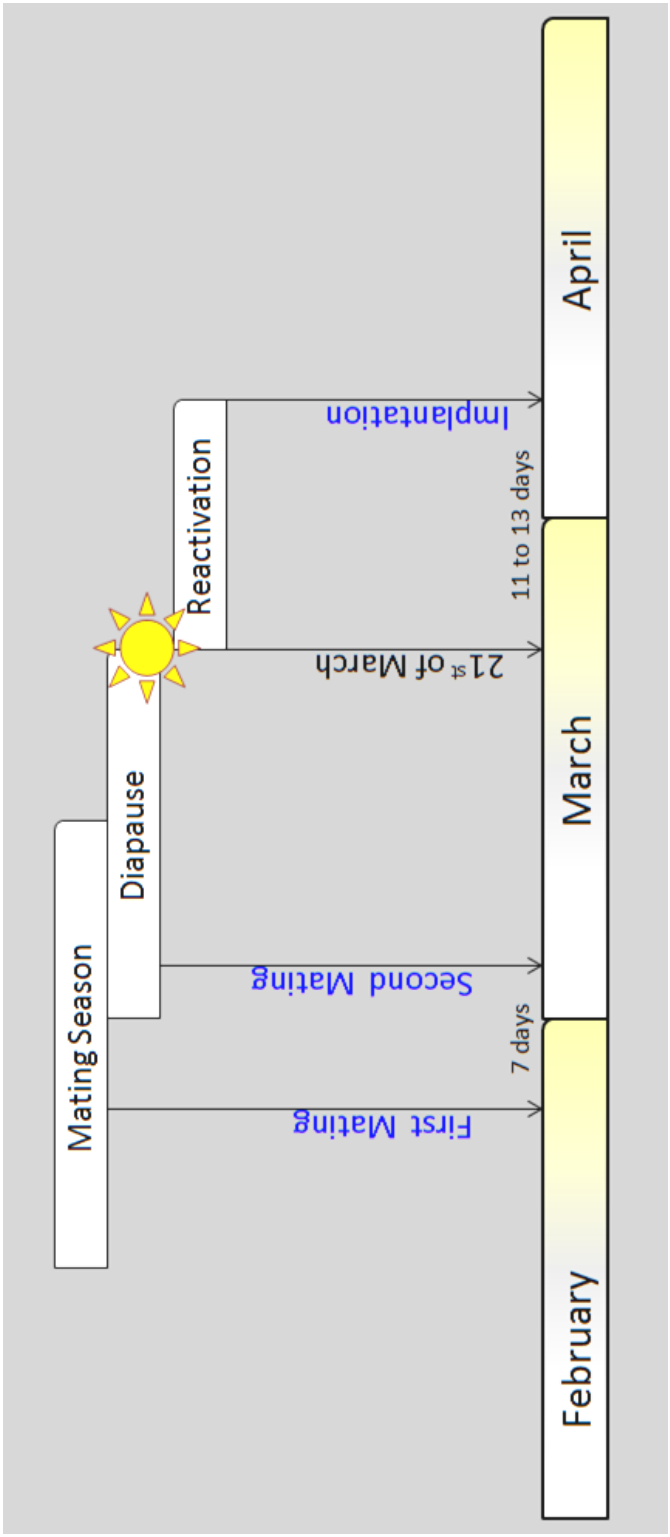

April
March
February
Mating Season
Diapause
Reactivation
First Mating
7 days
Second Mating
21st of March
11 to 13 days
Implantation

Figure 1: Schema of the Mink Season: Mating, Diapause, Reactivation, and Implantation. The mating season of the mink begins every year at the end of February. Following an experimental protocol the female minks are mated for a second time after 7 days. Embryos from the first and second mating will enter diapause. The reactivation of the embryo begins, with some individual variation when the photoperiod changes, the 21st of March. Following 11 to 13 days of reactivation, depending from the length of the photoperiod or use of prolactin, implantation occurs.

Reactivation	Long diapause	Diapause	Entry to diapause	**Groups**
4	8	4	5	**Numb of Females**
Matting	Matting	Matting	Matting	**11-Mar**
				12-Mar
				13-Mar
				14-Mar
				15-Mar
			Collection	**16-Mar**
				17-Mar
				18-Mar
				19-Mar
	Melatonin Implants			**20-Mar**
Prolactin Reactivation		Collection		**21-Mar**
				22-Mar
				23-Mar
				24-Mar
				25-Mar
				26-Mar
				27-Mar
Collection				**28-Mar**
				29-Mar
				30-Mar
				31-Mar
				1-Apr
				2-Apr
				3-Apr
				4-Apr
				5-Apr
				6-Apr
				7-Apr
				8-Apr
				9-Apr
				10-Apr
				11-Apr
				12-Apr
				13-Apr
				14-Apr
	Collection			**15-Apr**

Figure 2: **Schema for Embryo Collection in the Mink Season 2011: Matings, Treatments, and Embryo Collection in Four Mink Groups.** Embryos from the first group of 5 female mink, entry to diapause, were collected 5 days after second mating. Embryos from the second group of 4 females, in diapause, were collected 10 days after second mating. Embryos from 8 female mink from the long diapause group, implanted with melatonin implants from the 20th of March to the 15th of April, were collected the 15th of April. The embryos from the last group of 4 females, reactivated group, were collected after 7 days of prolactin injections to induce reactivation, the 28th of March. Yellow square: Days of mating, Green square: Days of treatment with prolactin for reactivation, Blue square: Days with melatonin implant, and Red square: Days of euthanasia and embryo collection.

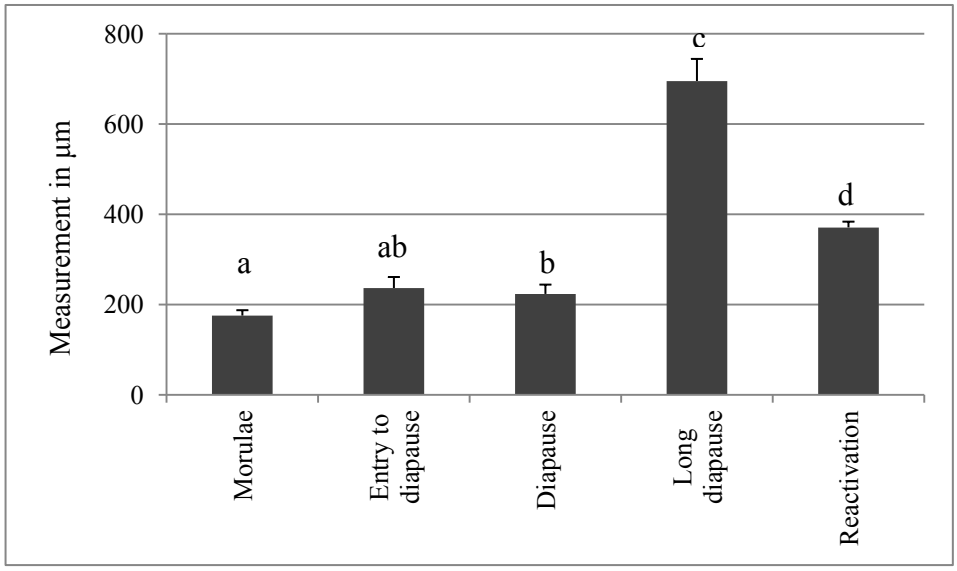

Measurement in µm
800
600
400
200
0
a
ab
b
c
d
Morulae
Entry to diapause
Diapause
Long diapause
Reactivation

Figure 3. Embryo Diameter of Mink Embryos during Different Stages between Entry to Diapause and Reactivation

Histograms represent the mean ± SEM of the embryo diameters in μm collected at different time points between entry to diapause and reactivation from the experimental groups described in Figure 1. Embryos in morulae stage were collected in the first two groups, entry to diapause and diapause, and represented as the first histogram. Embryos in blastocyst stage were collected in the other groups and represented in different histograms. Different superscripts indicate significant differences at $p < 0.0001$.

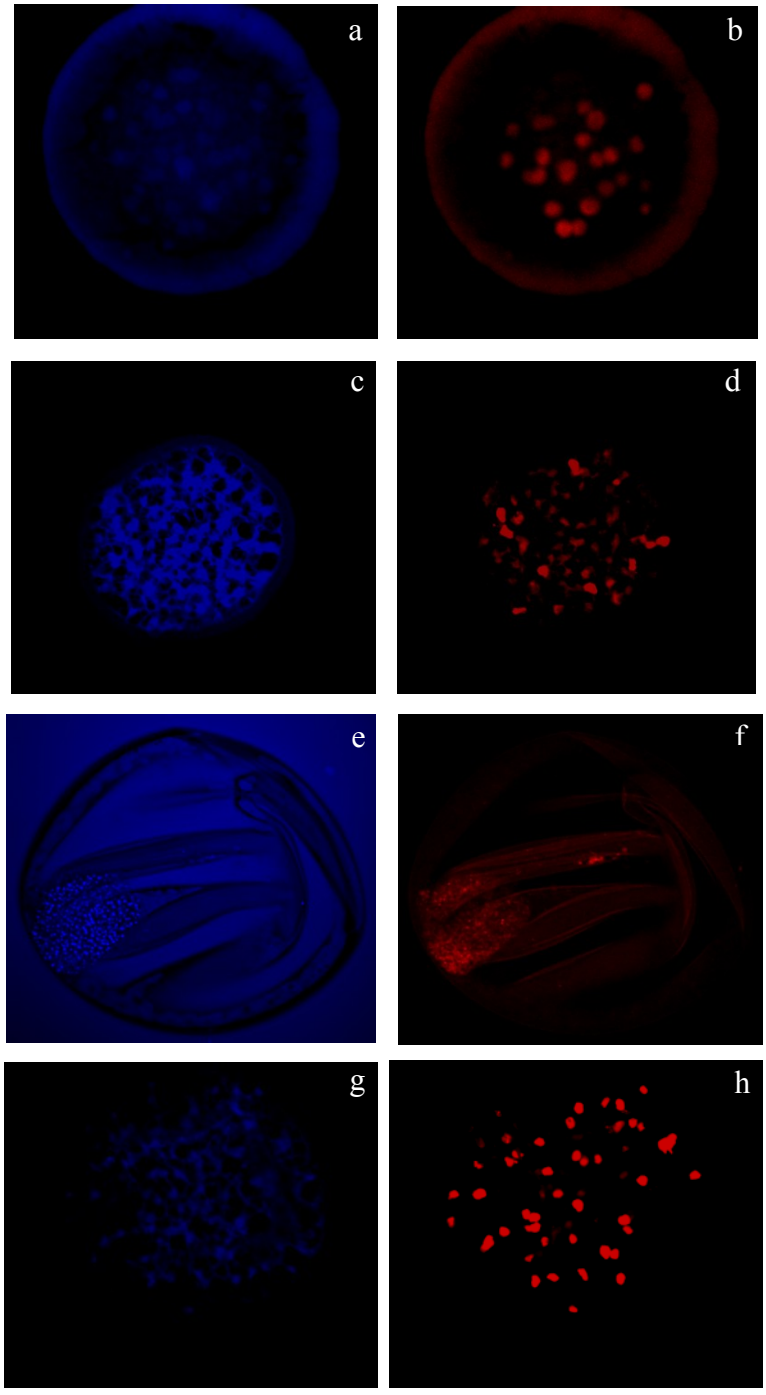

a
b
c
d
e
f
g
h

Figure 4. Mink Blastocyst Embryos during Different Stages between Entry to Diapause and Reactivation Labeled for Replication Experiment.

Blastocyst cells labeled with Hoechst 33342 are depicted in panels a, c, e, and g. Embryos labeled with Alexa Fluor 594 attached to 5-ethynyl-2'-deoxyuridine (EdU) can be found in panels b, d, f, and h. Panels a and b are embryos at entry to diapause, a blastocyst at diapause in c and d, a blastocyst in long diapause in e and f, and a reactivated blastocyst in g and h. Embryo photomicrographs of entry to diapause, diapause, and reactivation were taken at 200x magnification, while the photomicrograph of long diapause embryo was taken at 100x magnification.

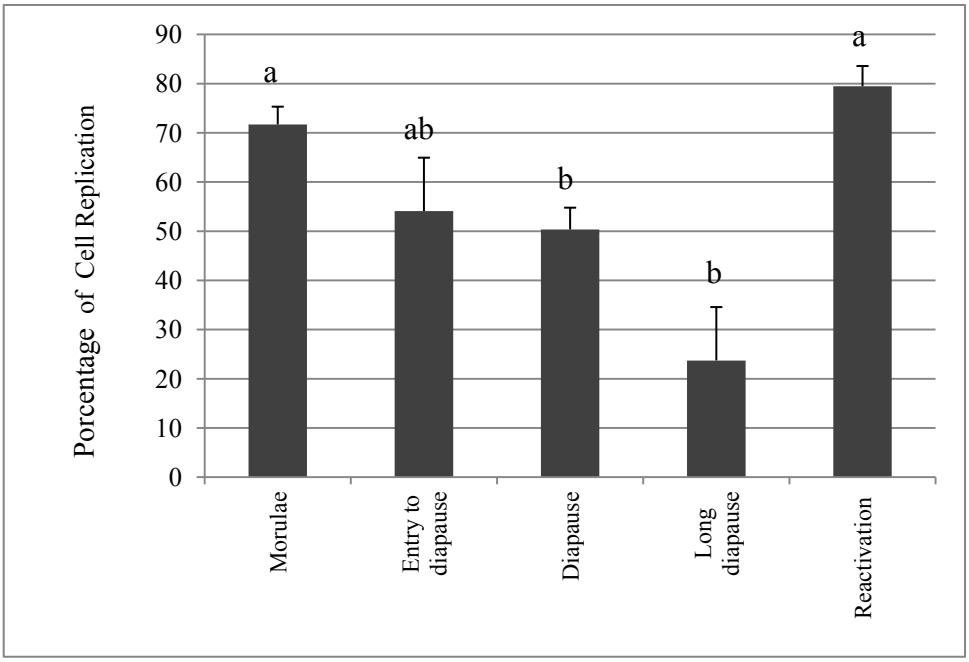

Porcentage of Cell Replication
90
80
70
60
50
40
30
20
10
0
a
ab
b
b
a
Morulae
Entry to diapause
Diapause
Long diapause
Reactivation

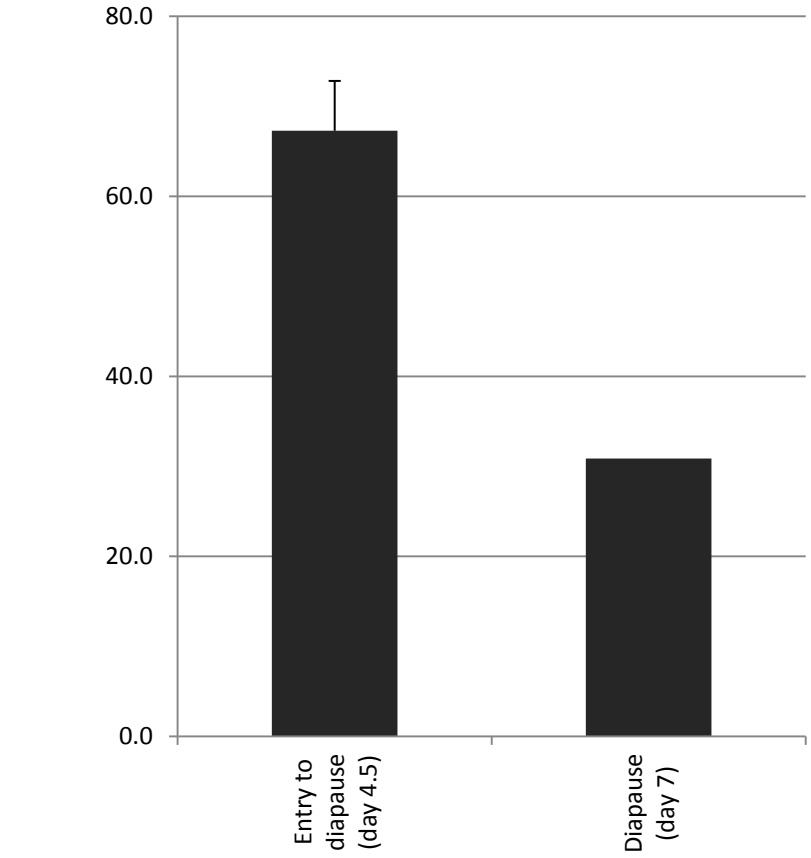
Figure 5. Percentage of Cell Replication in the Mink Embryos during Different Stages between Entry to Diapause and Reactivation

Histogram representing the mean ± SEM of percentage of cell replication from embryos collected at different time points from the experimental groups described in Figure 1. Embryos in morulae stage were collected in the first two groups, entry to diapause and diapause, and represented as the first histogram. Embryos in blastocyst stage were collected in the other groups and represented in separate histograms. Different superscripts indicate significant differences at $p = 0.006$.

Groups	d0	d1	d2	d3	d4	d5	d6	d7	d8	d9	d10	d11
Entry to diapause (day 4.5)	M	P		S								
Diapause (day 7)	M	P		S								
Long Diapause (day 11)	M	P		S								
Reactivation (day 9)	M	P		S					E			

Figure 6: Schema for Mating, Surgery, Treatments, and Embryo Collection of Four Groups of Mouse Embryos at Different Stages between Entry to Diapause and Reactivation

This scheme depicts the ideal experiment but embryos were acquired just from the first two groups. From the first group, entry to diapause, 7 embryos were collected one day after surgery. From the second groups, the diapause group, 2 embryos were collected 4 days after surgery. M: day of mating, P: Day of plug considered as day 1 post coitum (p.c.), S: day of ovariectomy, E: estradiol injection, squares in red: day of euthanasia, squares in blue: days of progesterone treatment.


80.0
60.0
40.0
20.0
0.0
Entry to diapause (day 4.5)
Diapause (day 7)

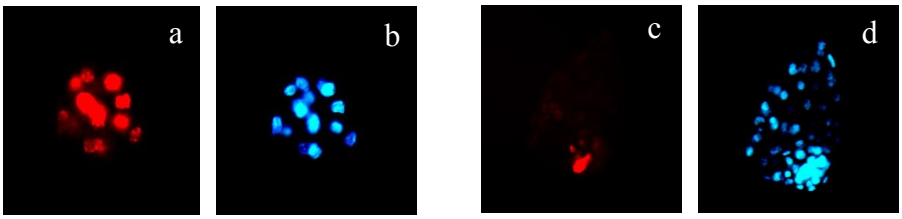
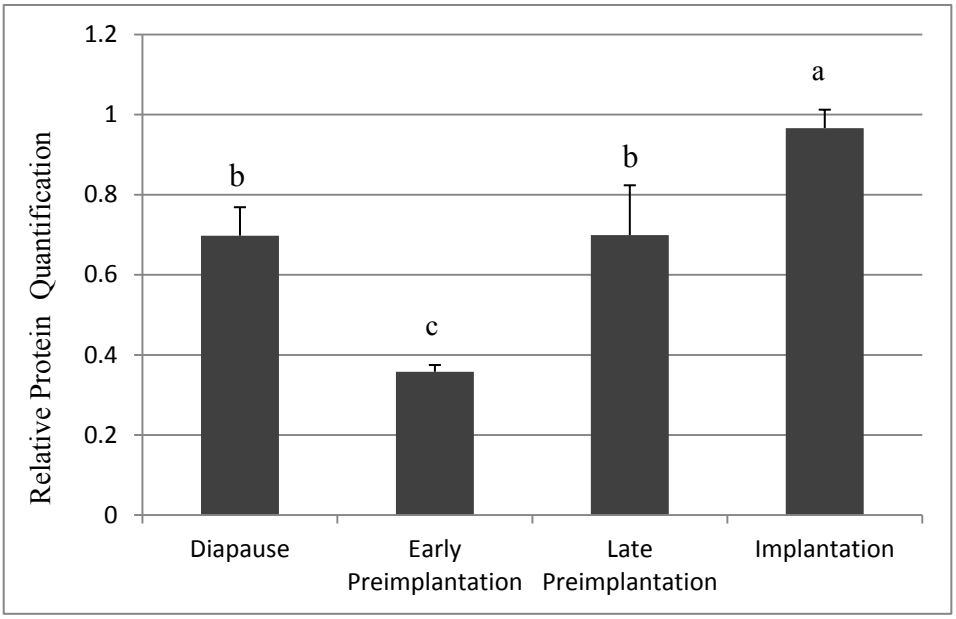

a
b
c
d

Figure 7. Replication in the Mouse Embryos at Entry to Diapause and Diapause.

Histograms represent the mean ± SEM of percentage of cell replication from embryos collected at two time points (entry to diapause and diapause). Superscripts indicate significant difference at $p < 0.05$. An embryo in the morulae stage, at entry to diapause, can be found in panels a and b, a blastocyst embryo at diapause can be found at panels c and d. Embryos labeled with Hoechst 33342 are in panels a and c. Embryos labeled with Alexa Fluor 594 attached to EdU are in panels b and d. All photomicrographs were taken at 400x magnification.

A)


1.2
1
0.8
0.6
0.4
0.2
0
Relative Protein Quantification
b
c
b
a
Diapause
Early Preimplantation
Late Preimplantation
Implantation

B)

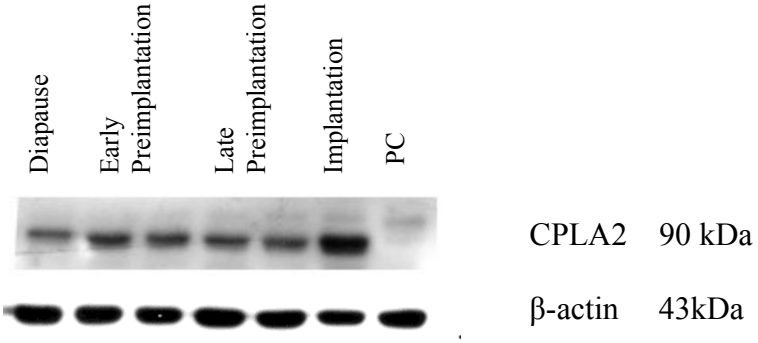

Diapause
Early Preimplantation
Late Preimplantation
Implantation
PC
CPLA2 90 kDa
β-actin 43kDa

Figure 8. Quantification of Cytoplasmic Phospholipase A2 (CPLA2) Protein in Mink Uterus from Diapause to Implantation

Total protein quantification of CPLA2 in mink uterus samples from diapause, early preimplantation, late preimplantation, and implantation. Histograms show ratios of CPLA2 normalized to β-actin for protein loading correction. Data represents the mean ± SEM of 3 different samples per stage. Superscripts indicate significant differences at $p < 0.05$. PC: positive control.

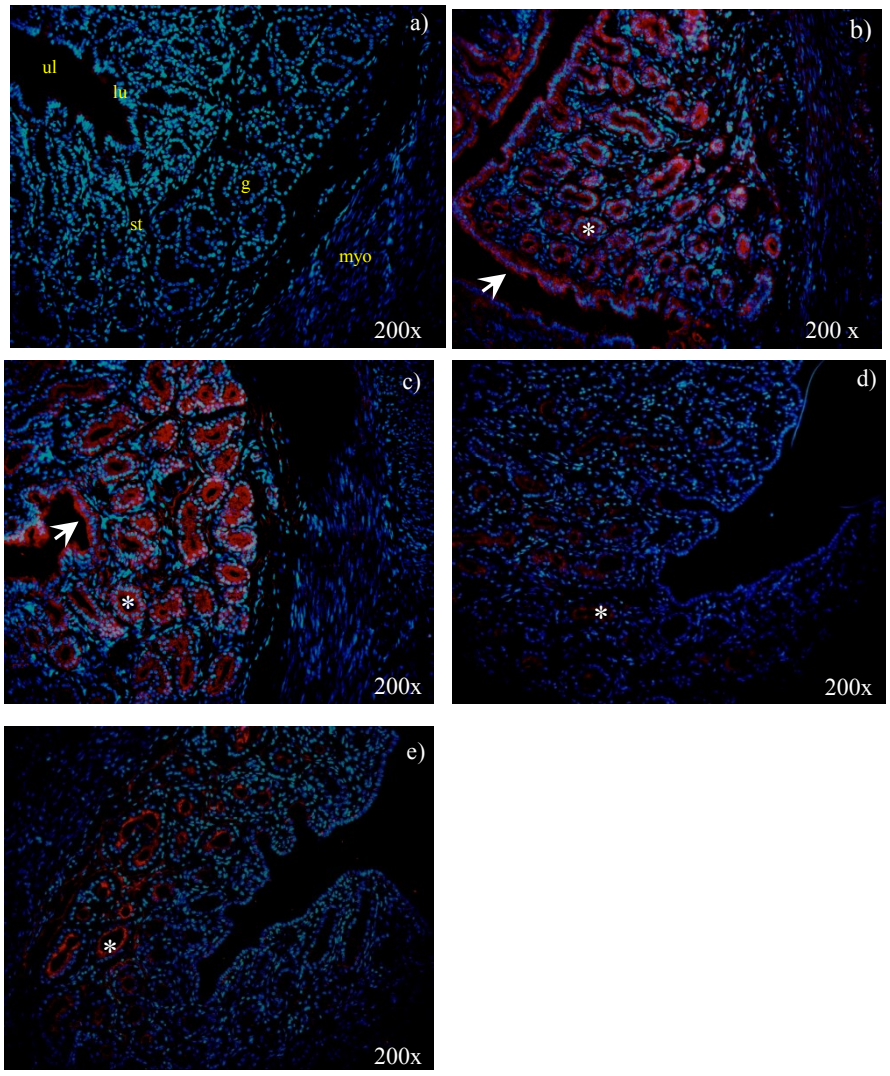
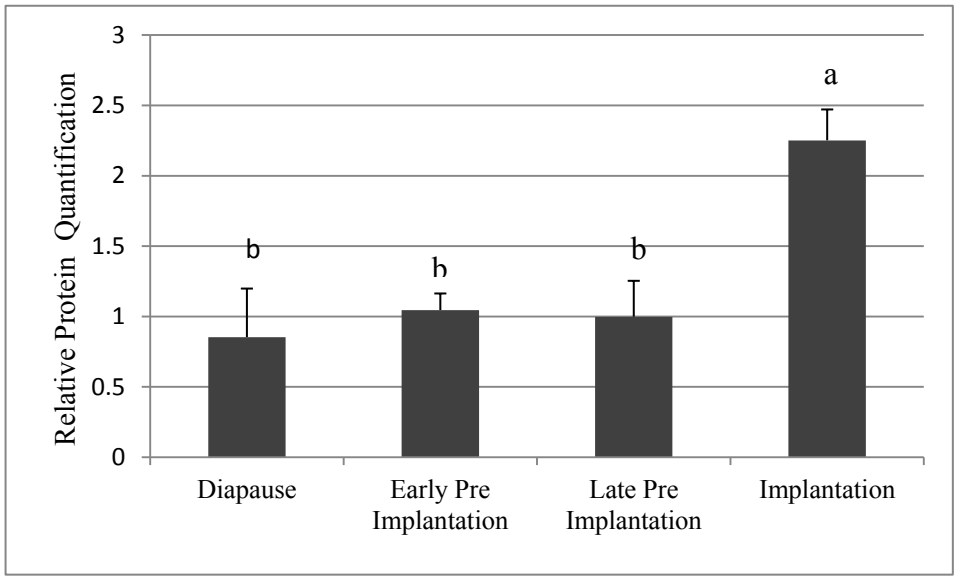

a)
ul
lu
g
st
myo
200x
b)
200 x
c)
200x
d)
200x
e)
200x

Figure 9. CPLA2 Immunofluoresence in Mink Uterus from Diapause to Implantation.

Protein localization of CPLA2 was performed in mink uterine tissue sections at diapause (b), in early preimplantation(c), at implantation sites (d), and at inter-implantation region (e). A tissue section from early preimplantation (a) was used as a negative control. Note the protein localization in the luminal epithelium and the glands. White arrows indicate the protein localization at the luminal epithelium, asterisks indicate the protein localization at the glands. ul: uterine lumen; g: gland; lu: luminal epithelium; myo: myometrium; st: stroma. All the figures correspond to 200x magnification.

A)


Relative Protein Quantification
3
2.5
2
1.5
1
0.5
0
b
b
b
a
Diapause
Early Pre Implantation
Late Pre Implantation
Implantation

B)


Diapause
Early PreImplantation
Late Preimplantation
Implantation
COX2
74kDa
ß-actine
43kDa

Figure 10. Quantification of Cyclooxigenase 2 (COX2) Protein in Mink Uterus from Diapause to Implantation

Total protein quantification of COX2 in mink uterus from diapause, early preimplantation, late preimplantation, and implantation. Histograms show ratios of COX2 normalized to β-actin for protein loading correction. Data represents the means ± SEM of 3 different samples per stage. Superscripts indicate means that are significantly different at $p < 0.05$. PC: positive control.

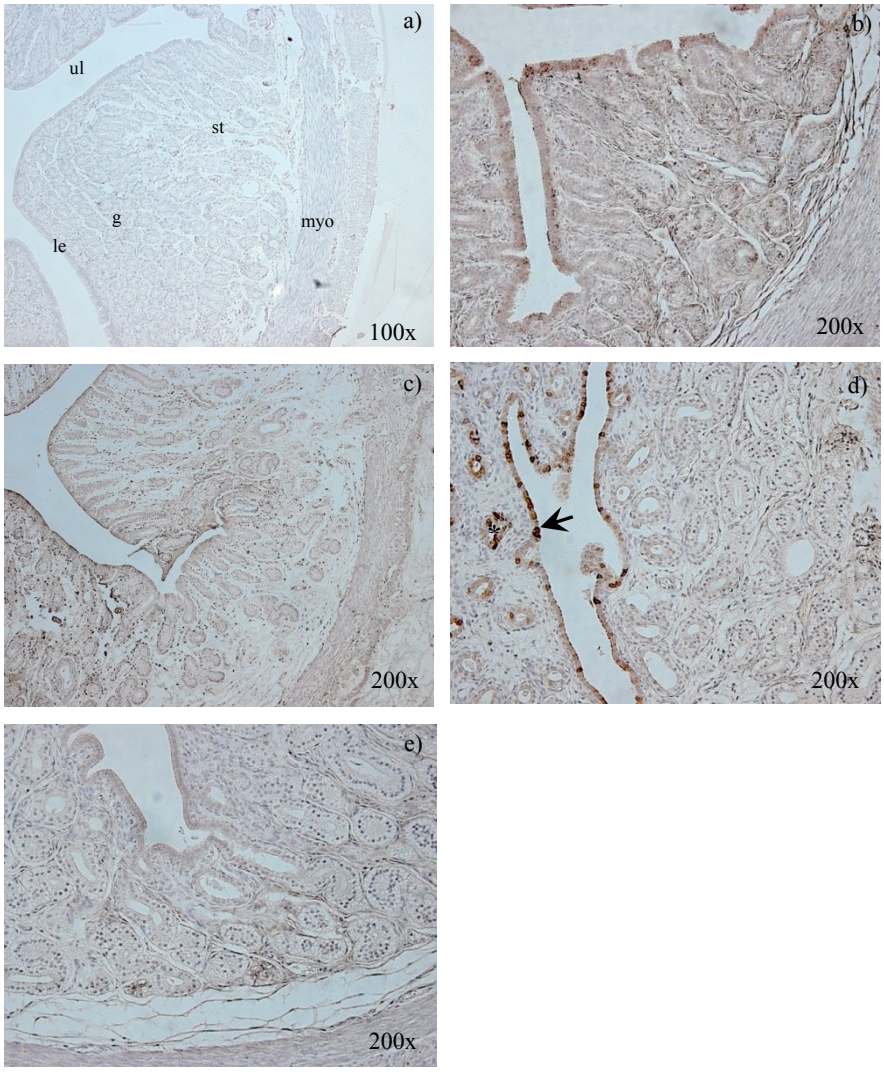

a)
ul
st
g
myo
le
100x
b)
200x
c)
200x
d)
200x
e)
200x

Figure 11. COX2 Immunohistochemistry in Mink Uterus from Diapause to Implantation.

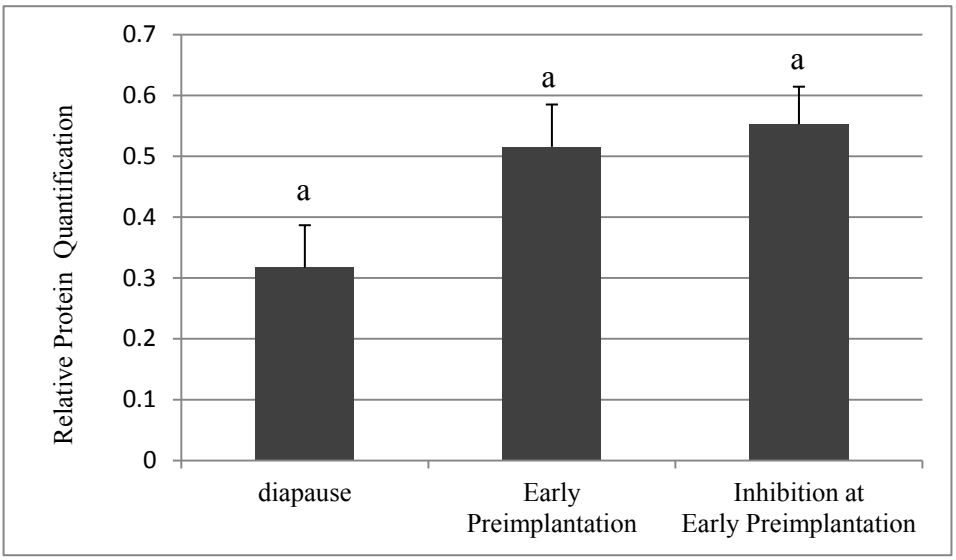
Protein localization of COX2 was performed in mink uterine tissue sections from diapause (b), early preimplantation (c), implantation (d), and at inter-implantation sites (e). A tissue section from diapause (a) was used as a negative control. Note the protein localization in the luminal epithelium and the glands (d). Black arrow indicates the protein localization at the luminal epithelium, asterisk indicate the protein localization at the glands. ul: uterine lumen; g: gland; le: luminal epithelium; myo: myometrium; st: stroma. All the figures correspond to 200x magnification.

Groups	Diapause	Early Preimplantation	Inhibition at Early Preimplantation
Numb of Females	5	5	5
11-Mar	Matting	Matting	Matting
12-Mar			
13-Mar			
14-Mar			
15-Mar			
16-Mar	Collection		
17-Mar			
18-Mar			
19-Mar			
20-Mar			
21-Mar		Prolactin Reactivation	Prolactin reactivation + Meloxicam inhibition of COX2
22-Mar			
23-Mar			
24-Mar			
25-Mar			
26-Mar			
27-Mar			
28-Mar		Collection	Collection

Figure 12: Scheme of Meloxicam Experiment: Matings, Treatments, and Uterus Collection in 3 Groups of Female Minks.

Uterine samples were collected from female mink at different time points. Uterine samples from the diapause group, 5 females, were collected 5 days after mating. Prolactin injection was performed in two female mink groups, early preimplantation and inhibition at early preimplantation, for uterus reactivation. Uterus samples from the second group, early preimplantation used as a control for COX2 inhibition, were collected after 8 days of prolactin injections. Meloxicam treatment was performed for COX2 inhibition. Uterus samples from the third group, inhibition at early preimplantation, were collected after 8 days of prolactin and Meloxicam injections.

A)


Relative Protein Quantification
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
a
diapause
Early Preimplantation
Inhibition at Early Preimplantation

B)

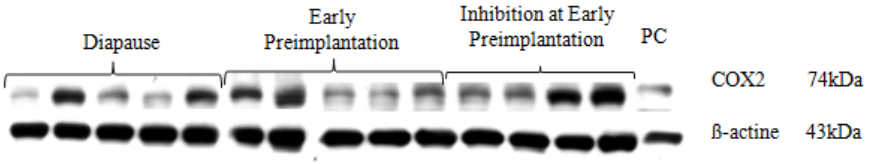

Diapause
Early Preimplantation
Inhibition at Early Preimplantation
PC
COX2 74kDa
ß-actine 43kDa

Figure 13. Effect of Meloxicam Treatment in the Quantification of COX2 in Mink Uterus during Diapause and Early Preimplantation.

Total protein quantification of COX2 from mink uterus at diapause, at early preimplantation, and at early preimplantation and COX2 inhibited with Meloxicam. Histograms show ratios of COX2 normalized to β-actin for protein loading correction. Data represent the mean ± SEM of 5 different samples for diapause, 4 samples for early preimplantation, and 5 for inhibition at early preimplantation. Different superscripts indicate differences at $p < 0.05$. PC: positive control.

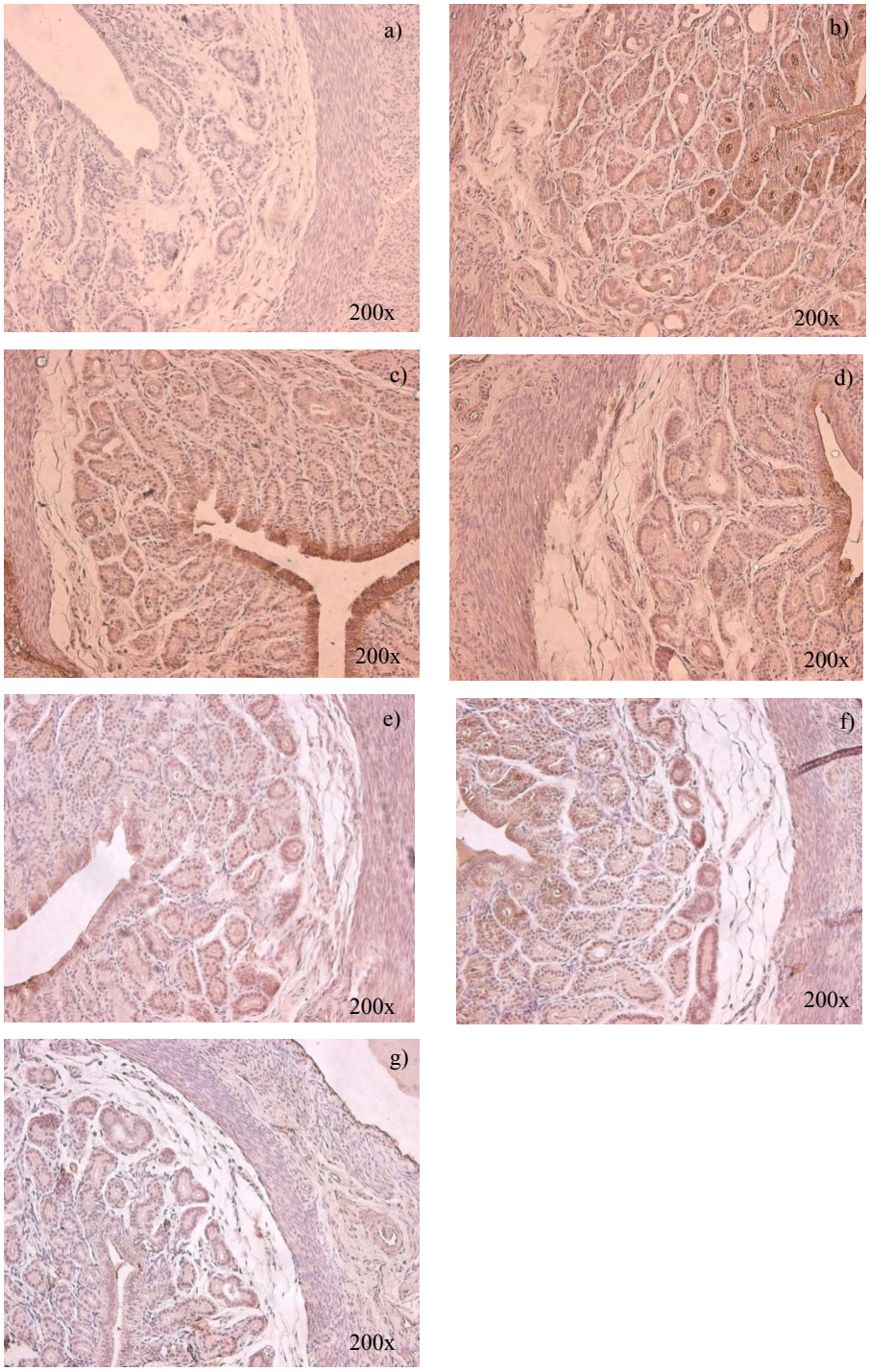
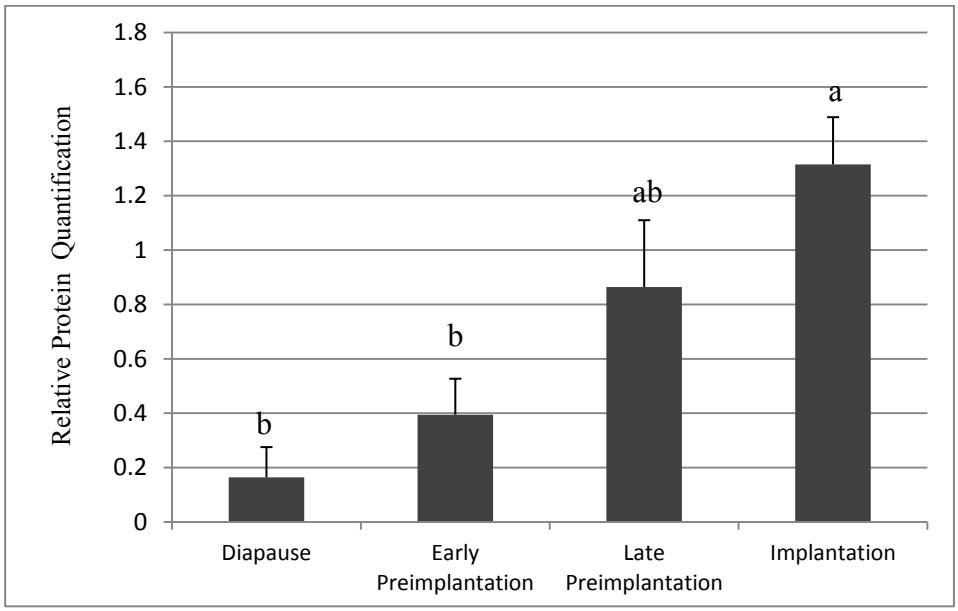

a)
200x
b)
200x
c)
200x
d)
200x
e)
200x
f)
200x
g)
200x

Figure 14. Effect of Meloxicam Treatment in COX2 Immunohistochemistry in Mink Uterus during Diapause and Early Preimplantation. Protein localization of COX2 was performed in mink uterine tissue sections at diapause (b,c), early preimplantation (d,e), and early preimplantation following inhibition with Meloxicam (f,g). A tissue section from diapause (a) was used as a negative control. All the figures correspond to 200x magnification.

A)


Relative Protein Quantification
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
b
b
ab
a
Diapause
Early Preimplantation
Late Preimplantation
Implantation

B)

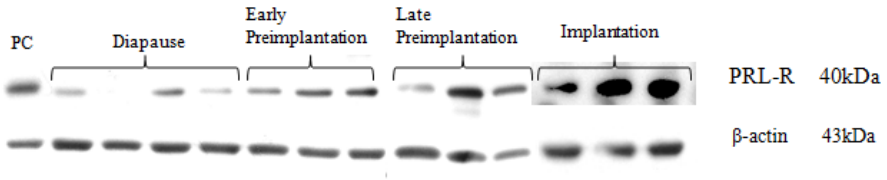

PC
Diapause
Early Preimplantation
Late Preimplantation
Implantation
PRL-R 40kDa
β-actin 43kDa

Figure 15. Prolactin Receptor (PRL-R) in Mink Uterus from Diapause to Implantation

Total protein quantification of PRL-R in mink uterus from diapause, early preimplantation, late preimplantation, and implantation. Histograms show ratios of PRL-R normalized to β-actin for protein loading correction. Data represents the mean ± SEM of 3 different samples. Means bearing different superscripts are significantly different at $p < 0.05$. PC: positive control.

DISCUSSION:

Since the discovery of delayed implantation in 1854 in the roe deer (reviewed in (Isakova 2006), the phenomenon of embryonic diapause has intrigued researchers. It has elements to suggest that it may serve to aid in understanding the cross talk between the embryo and the uterus (Dey, Lim et al. 2004) and particularly for the identification of molecules for embryo implantation and uterine receptivity.

In the mink, the timing of implantation, and therefore the length of delay, is related to the changing photoperiod around the 21st of March which has the effect of decreasing the diurnal duration of melatonin secretion (Ravault, Martinet et al. 1986). The decrease of melatonin triggers the secretion of prolactin (Murphy, DiGregorio et al. 1990). Prolactin, the luteotrophic factor in the mink (Murphy, Concannon et al. 1981), reactivates the corpus luteum (CL) to increase the secretion of progesterone. As a consequence, progesterone activates the uterus and the embryo and induces implantation (Lopes, Desmarais et al. 2004). The process of diapause can be examined at three arbitrary time points: entry into diapause, diapause itself, and reactivation. Little is known about what happens to the embryo during the entry into diapause, in comparison with the activation (Desmarais, Bordignon et al. 2004). In this study, it was shown that there is an elevated rate of replication in the morulae and that this rate becomes reduced as the embryo enters diapause.

Reactivated mink blastocyst growth coincides with the increase of progesterone and the embryo diameter is related to the time of reactivation (Martinet, Allais et al. 1981; Desmarais, Bordignon et al. 2004). This is also been showed in another mustelid, the spotted skunk (*Spilogale pygmaea)* (Enders, Schlafke et al. 1986). In the case of the spotted skunk, a mustelid that shows a longer diapause, there is a slow increase of approximately 60% in blastocyst size over diapause (Enders, Schlafke et al. 1986). Melatonin treatment in the mink can induce longer diapause (Murphy, DiGregorio et

al. 1990). In the present study, embryos from long diapause induced with melatonin were isolated and presented a larger diameter in comparison with the diapause embryos and the reactivated ones. The presence of dividing cells in these embryos argues that their viability is not compromised. However, the cause of the much greater diameter from these embryos is not known. It is believed that fluid uptake could happen during long diapause, but further studies will be necessary for confirmation.

It was believed that division of cells of the blastocyst during diapause occurs at a very low rate or that the cells of the blastocyst are totally quiescent. For instance, trophoblast cells from the roe deer have a low rate of proliferation (Lengwinat and Meyer 1996), kangaroos and wallabies have no growth in the blastocyst during diapause (Renfree and Callaby 1981; Smith 1981), and mink embryos were believed to be in G1/S cell cycle arrest (Desmarais, Bordignon et al. 2004). However in the present study, it was shown that cell replication in mink embryos occurs, not only during diapause, but also when diapause was artificially extended with melatonin. In the method we employed, tracking the uptake of DNA precursors it was not possible to distinguish whether cytokinesis was completed, or whether the DNA synthesis is indicative of endoreduplication (Isakova and Mead 2004; Isakova 2006). Previous unpublished studies (Lefèvre P. 2010) suggested that there is down regulation of the thioredoxine (TRX) gene believed to be essential for proliferation (Yoshida, Nakamura et al. 2005). It is known that TRX (-/-) mouse embryos show embryonic lethality due to failure in zona pellucida (ZP) hatching and trophoblast attachment (Matsui, Oshima et al. 1996), and the absence of hatching and trophoblast attachments are characteristics of mink embryos during diapause.

Following our results in mink embryos, delayed implantation was induced in mice to compare obligate diapause with facultative diapause. The results showed that, as in the mink, DNA replication occurs during diapause. Diapause in the mouse embryo has been attributed to CyclinD1, CyclinE1, and CyclinE2 down regulation and $p21^{Cip1/WAF1}$ up regulation in dormant

mouse embryos (Hamatani, Daikoku et al. 2004). Other authors have suggested that there is over expression of p21 and this has the effect of preventing apoptosis (Ullah, Lee et al. 2009; Ullah, Lee et al. 2009) during diapause. Mechanisms aside, the data together suggest that mitosis occurs in embryos during diapause for both the mink and the mouse.

The concept of crosstalk for implantation suggests that the uterus signals to the embryo and viceversa. The uterus needs to prepare for the attachment and to provide the necessary molecules to reactivate embryo development. In many species, the endometrium of the uterus undergoes a process of vascularization and decidualization associated with implantation and requiring prostaglandins (Kennedy, Gillio-Meina et al. 2007). While it is known that the mink endometrium does not present decidualization, there are some similarities, as prostaglandin E2 (PGE2) is expressed in mink uterus prior and around the time of implantation (Lopes, Desmarais et al. 2006). It therefore is plausible to suggest that uterine prostaglandins are required for termination of diapause in mink.

The first molecule necessary for PG biosynthesis from cell membrane phospholipids is cytosolic phospholipase 2 (CPLA2) as shown in bovine endometrial cells (Godkin, Roberts et al. 2008). CPLA2 expression increases around the time of implantation in rats (Novaro, Rettori et al. 1996) and in mouse at implantation and postimplantation in epithelial and stromal cells respectively (Song, Lim et al. 2002). The data in the present study show that CPLA2 increases at the protein level at implantation in comparison with the other stages and that it localizes to the luminal epithelium and the glands. However, during the early preimplantation stage we found CPLA2 concentration was reduced compared to diapause and late pre-implantation, suggesting that another protein from the same family could be acting during this short period. In the case of the human cells MCF-7, a band close to 100 kDa was expected (Pacurari 2006); and the low intensity of band could be due to the fact that protein isolation was

performed from 0.56 x 10^6 cells and aliquoted in 200 ul tubes without taking in consideration the quantity of total protein.

Secretory phospholipase (SPLA2) and CPLA2 are functionally coupled with cyclooxygenase (COX) for PG biosynthesis (Murakami, Nakatani et al. 2000). COX1 and COX2 convert arachidonic acid into prostaglandin H2 (PGH2), common substrate for the PG biosynthesis. While COX1 is a constitutive protein, COX2 is inducible (Pakrasi and Jain 2008). In the mouse, COX2 localizes in the stroma cells after implantation takes place (Song, Lim et al. 2002). In the western spotted skunk, COX2 was not expressed during diapause but it was detected prior to attachment and remained detectable for 5 to 6 days in the luminal and glandular epithelium (Das, Wang et al. 1999). In the present study, COX2 protein in mink uterus increases at implantation and is located in the luminal epithelium and glands suggesting a co-localization at the time of implantation with cPLA2 protein. However, double antibody immunohistochemistry will be necessary to further explore this idea.

Taking in consideration the importance of COX2 for embryo implantation, we attempted to address its role by inhibiting its activity. Meloxicam is considered a selective inhibitor of COX2, but not COX1, activity (Fosslien 2000), shown in sheep (Rac, Scott et al. 2007), was used. The western blot results from COX2 enzyme activity inhibition with Meloxicam in mink uterus from diapause, reactivation and inhibition of reactivation did not show any difference at the protein concentration. This could be explained with the fact that Meloxicam did not affect the protein synthesis rather it affected the activity of COX2. To determine whether the activity was inhibited with Meloxicam and taking in consideration that COX2 converts arachidonic acid into prostaglandin H2 (PGH2), common metabolite for the PGs. PGE2 and PGFα concentration were analyzed between the three groups by HLPC. Results from HPLC (data not shown) showed no difference in PGE and PGFα suggesting that COX2 activity inhibition did not affect the prostaglandin synthesis. Another member of the prostaglandin

synthesis is COX1, taking in consideration this the PGs observed could be due to COX1 activity and not COX2 activity necessarily. However, we should take in consideration from the previous COX2 western blot results at the preimplantation, that COX2 has a peak of synthesis at implantation. Further studies inhibiting COX2 activity for 13 days and determination of the effects on the abundance of metabolites from this pathway, as PGI, PGFα, and PGE are required.

From the western blot data for prolactin receptor (PRL-R) in the present study, is it clear that PRL-R abundance increased during the period of treatment with PRL and activation of the embryo. It has been shown that in rat stroma cells PGE2 is stimulated by PRL in a dose dependent manner by increasing SPLA1 and COX2 (Prigent-Tessier, Pageaux et al. 1996). Prolactin binding sites were demonstrated in the luminal and glandular epithelium of the ferret uterus (Rose, Huang et al. 1993). In the case of the mink, it was suggested that prolactin could upregulate its own receptors in the mink uterus (Rose, Slayden et al. 1996). This study confirms the increase of prolactin receptors from diapause through implantation and confirms the idea of prolactin upregulating its own receptor at the uterus. However, further studies showing a link between the prolactin receptors and proteins related to the PG pathway will be necessary.

CONCLUSIONS:

It is evident that the process of diapause is not related to a complete cell cycle arrest since DNA replication was observed in mink embryos during diapause and long diapause. We saw that DNA replication goes down from early blastocyst to diapause and then it goes up with reactivation. While this happens, the photoperiod changes and progesterone in combination with prolactin may reactivate the uterus, with the latter acting through its own receptor. The consequence is that at the same time, CPLA2 and COX 2 are up regulated to produce prostaglandins required for angiogenesis and implantation.

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