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**The effects of estradiol and progesterone on uterine glycogen
metabolism in the American mink (*Neovison vison*)**

**By
Kole Justin Bowman**

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To the Graduate Faculty:

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Abstract

Mink were treated with E₂, P₄ or vehicle (control) for three days and uteri collected 24 h (E₂, P₄ and vehicle) and 96 h (E₂) later. Additionally, mink were treated first with E₂ followed by P₄, each for three days (E₂→P₄) with uteri collected 24 h later. Percent glycogen content in response to E₂ was greater at 96 h than 24 h, and both were higher than controls. E₂→P₄ treatment reduced glycogen content, while concomitantly increasing catabolic enzyme (glycogen phosphorylase and glucose-6-phosphatase) mRNA expression and amount of phospho-glycogen synthase protein (inactive) in uterine homogenates. Interestingly, E₂→P₄ increased glycogen synthase mRNA and hexokinase (mRNA and protein) production. To us, this suggests that P₄ stimulates uterine glycogen catabolism after mating, releasing glucose that supports pre-embryonic survival and development. During implantation, E₂ and P₄ stimulate glycogen synthesis (diverting maternal glucose to the uterus) and catabolism ensuring an adequate source of energy for the establishment of pregnancy.

Introduction

Pre-embryonic growth, implantation and early fetal development are dependent upon uterine glandular and luminal epithelial cell secretions (histotroph) containing glucose, glycogen, amino acids, fats, ions and hormones (Burton et al., 2002; Gray et al., 2001b; Hempstock et al., 2004). Although gluconeogenesis does not occur in the uterus (Yáñez et al., 2003; Zimmer and Magnuson, 1990) glucose is taken up, transported into the uterine lumen, metabolized as a fuel source or when in excess, stored as glycogen.

Glycogen synthesis begins with phosphorylation of glucose by hexokinase (Hk), producing glucose-6-phosphate, which is isomerized to glucose-1-phosphate and converted to uridine diphosphate glucose (Ferrer et al., 2003). Glucosyl units from the latter are transferred to non-reducing ends of glycogen molecules by glycogen synthase (Gys). Glycogen is catabolized by glycogen phosphorylase (Pyg), releasing glucose-1-phosphate, which may be isomerized to glucose-6-phosphate and enter the glycolytic pathway, or be dephosphorylated by glucose-6-phosphatase (G6pc), releasing glucose for export to the systemic circulation and/or uterine lumen.

Uterine glycogen content in rodents peaks during proestrus to estrus, and is mobilized during implantation and pregnancy (Demers et al., 1972; Greenstreet and Fotherby, 1973a). Similarly, the human uterus stores large amounts of glycogen during the late proliferative to early secretory phases, which is mobilized by the late secretory phase (Cornillie et al., 1985; Spornitz, 1992; Verma, 1983). We have shown that uterine glandular and luminal epithelial glycogen content of the mink decreased 99% between estrus and the peri-implantation period (Dean et al., 2014). Mink are seasonal breeders exhibiting obligatory embryonic diapause and may have blastocysts (as many as 17) in a

state of arrested development for 50-60 days *post coitum*, resulting in delayed implantation (Enders, 1952; Hansson, 1947). It is likely that mink uterine glycogen reserves contribute to pre-embryonic survival, implantation and perhaps the number of offspring per litter.

Uterine glycogen synthesis is stimulated by estradiol (E_2) in rats, rabbits, guinea pigs (Demers et al., 1972, 1973a, 1973b; Greenstreet and Fotherby, 1973b) and mink (Rose et al., 2011). In the ovariectomized rat, exogenous E_2 increased uterine glycogen content as expected (Paul and Duttagupta, 1973). However, when treated concomitantly with E_2 and progesterone (P_4), uterine glycogen concentrations were reduced when compared to E_2 alone. Demers and Jacobs (1973) treated ovariectomized rabbits and guinea pigs with E_2 followed by P_4 ($E_2 \rightarrow P_4$) to simulate E_2 priming and reported a reduction in uterine glycogen content and a two-fold increase in glycogen phosphorylase activity, compared to E_2 alone. These findings suggest that P_4 promotes uterine glycogen catabolism and/or antagonizes the glycogenic actions of E_2 .

Circulating E_2 concentrations in mink increase after mating, then decline during the variable period of embryonic diapause and increase again during the peri-implantation period (Lagerkvist et al., 1992). Plasma P_4 concentrations in mink remain low until the vernal equinox (regardless of the time of mating), then increase concomitantly with E_2 during the peri-implantation period. It is possible that E_2 promotes glycogen accumulation during proestrus and estrus, and that after mating and ovulation, P_4 triggers glycogen mobilization.

If this is true, the mink uterus must be extremely sensitive to P₄ at this time, since circulating concentrations of the steroid, while increasing, are very low until late diapause (Lagerkvist et al., 1992). Nevertheless, increased uterine sensitivity to P₄, in response to E₂ induced up regulation of P₄ receptors, may result in a uterus that responds to low (perhaps unchanging) P₄ concentrations. Such a phenomenon could promote glycogen mobilization, supporting the energy requirements of pre-embryonic development to the blastocyst stage.

LITARATURE REVIEW

The female American mink (*Neovison vison*) is a seasonal breeder that exhibits obligatory embryonic diapause, resulting in delayed implantation. As a result, the animal may have as many as 17 blastocysts up to 60 days of age at the time of implantation. This phenomenon makes the mink an excellent experimental model with which to investigate those uterine conditions that are optimal for implantation and therefore reproductive success. The findings from such experiments could lead to cures for infertility and perhaps safer and more efficient means of birth control.

Seasonal Reproduction is regulated by Photoperiod

Female mink on commercial ranches become receptive to males between late-February and mid-March (Enders, 1952; Hansson, 1947; Moyle pers comm.). The transition from anestrous to estrous, blastocyst reactivation and implantation occurs in response to the increasing day-length or photoperiod (Enders, 1952; Hansson, 1947; Moyle pers comm.). As evidence, exposure of anestrous mink (June 1st) to supplemental light (double the annual rate of increase and decrease) has been shown to advance the onset of estrus by three months, from the beginning of March to early December (Duby and Travis, 1972). Conversely, exposure of animals to shorter than normal photoperiods results in delayed luteal development (Mead, 1981). In a seminal experiment, Travis and Pilbeam (1980) abruptly switched the photoperiod to which mink were exposed from the ambient northern hemisphere to an artificial light regimen similar to the southern hemisphere. After a transition period of nine months, the mink appeared to adapt to the experimental photoperiod, exhibiting reproductive cycles that were six-months out of

phase with mink maintained under a northern hemisphere photoperiod. It is likely that the temporal pattern of reproductive cycles in mink, in response to the changing photoperiod evolved as a way to couple the birth of mink kits at a time of year that would be most conducive to survival (Murphy, 2012).

Photoperiodic Effects are mediated through the Pineal Gland and its Production of Melatonin

The photoperiod (ratio of light to dark) changes each day of the year, increasing during the spring and decreasing during the fall. Teleologically, this results in a mechanism/s by which the animal “knows” what time of the year it is. Seasonal changes in photoperiod not only determine the onset of estrus and implantation, but also trigger the onset of both summer and winter fur growth cycles in mink (Rose et al., 1984). As will be discussed, the hormone melatonin is secreted during the dark phase of each 24 h day (Reiter et al., 2014). Thus, during the spring, as the ratio of light to dark increases, the daily duration of melatonin secretion is gradually decreasing and the female mink become sexually receptive to males (enter estrus). While it might be logical to view melatonin as having negative effects (inhibiting anestrus to estrus transition) on reproduction, it is probably more accurate to view the temporal pattern of melatonin secretion as the trigger for onset of estrus-like behavior.

The eyes are the receptors for perceiving the daily photoperiod (light/dark), as one might predict. Recent findings show that light (460-480 nm) first impinges upon and activates intrinsically photoreceptive retinal ganglion cells (*ipRGC*) in the eyes (Reiter et al., 2014). The retinal ganglion cells transmit the received signal and release the

transmitters L-glutamate and pituitary adenylate cyclase-activating peptide, onto neurons of the suprachiasmatic nucleus (SCN). The SCN is viewed as an internal biological clock (circadian; Hadley and Levine, 2006) with an approximate rhythm of discharge of 24 h. During the dark phase of the 24 h day, the SCN activates neurons of the paraventricular nucleus, which communicates through the intermediolateral cell column in the thoracic cord. These neurons synapse with adrenergic neurons of the superior cervical ganglia, which finally synapse upon pinealocytes of the pineal gland. During the dark, increased adrenergic activity stimulates the synthesis of melatonin (MEL) by the pineal gland. Thus, the SCN is responsible for generating the nocturnal production of MEL. During the light phase, stimulation is inhibited and MEL synthesis and secretion decreases. As an additional mechanism, in some species (birds and amphibians) with thin skull bones, light has been shown to directly stimulate the neural circuitry of the brain (Cardinali et al., 1975; Korf, 1994).

In long-day breeders (mink), those that enter estrus as day-length increases, melatonin has been described as antigonadotrophic, as increased melatonin production during short days leads to reproductive quiescence (Reiter et al., 2009). Melatonin's activity appears to be stimulatory in short-day breeders however (sheep, deer and goats). In short-day breeders, increased melatonin production acts on the pituitary to increase gonadotropin-releasing hormone (GnRH) production, which stimulates the release of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH; Senger, 2005). The release of FSH and LH stimulate the growing follicle to develop and produce estrogens that induce estrus behavior.

Murphy et al. (1990) demonstrated melatonin's role in repressing reproductive activity in mink. Researchers treated mink with melatonin releasing implants. The following breeding season mink treated with MEL were mated, yet no embryos implanted. In mink, melatonin appears to decrease prolactin and progesterone secretion, both of which are essential for implantation and gestation. Melatonin treated mink that were subsequently given injections of prolactin resulted in increased progesterone secretion and embryo implantation. In these animals it appears that melatonin acts on the pituitary of mink to decrease prolactin secretion, yet it is unlikely that this represents the only action of MEL on reproduction in mink.

Ovulation is Induced by Mating

Mink are induced-ovulators, with ovulation occurring approximately 33-72 hours after mating (Enders, 1952; Hansson, 1947). The animals remain receptive to mating throughout the breeding season (late Feb to mid March) and will mate several times with the same or different males. This results in the phenomena of superfetation (having embryos of different ages present during the same pregnancy) and superfecundation (having offspring from the same cycle fertilized by different sexual acts). Based on this reproductive behavior, mink ranchers routinely mate mink 1-2 times during the first week of March and then remate them 1-2 times, after a 6-10 day interval. While this is done to maximize litter sizes, it is known that many of the fertilized eggs from the first mating are lost at the time of the second mating (after 6-10 days). Lagerkvist et al. (1992) suggested that the increase in litter size following a second mating is attributed to an increased number of ovulated eggs and a lower mortality rate of the blastocyst before implantation due to shorter gestation period.

Embryonic Diapause

Mink are reported to exhibit obligatory embryonic diapause. Following mating and fertilization of the eggs, the pre-embryo develops to the blastocyst stage of embryogenesis while it passes from the oviducts into the uterine lumen. At this time, the blastocyst enters a state of relative inactivity, wherein DNA, RNA and protein synthesis are reduced, resulting in a resting-like state called diapause.

Diapause, as well as seasonal estrus, are thought to be an evolutionary strategy to increase the likelihood of favorable conditions existing in the environment, to help ensure the survival of the offspring (Lindenfors et al., 2003). Embryonic diapause has been observed in over 130 mammal species, with differing modes of blastocyst re-activation and implantation (Fenelon et al., 2014). Even with the biochemical differences between the species, the study of embryonic diapause presents an opportunity to understand the minimum requirements for embryonic survival, and may later be used to increase fertility rates in humans and increase production in farm animals. Understandings of these processes could also be important in the further development of contraceptives that act through these pathways.

Embryonic diapause can either be obligatory or facultative in nature. Obligatory embryonic diapause has been observed in the American mink (*Neovison vison*; Murphy, 2012), Roe deer (*Capreolus capreolus*; Aitken, 1980) and the Western spotted skunk (*Spilogale gracillis*; Mead, 1981). These animals have embryos that must pass through a period of delayed development when metabolic activity of the embryo is decreased. These embryos remain dormant until reactivated by maternal signals (Mead, 1993). Facultative diapause is usually related to some form of metabolic stress placed on the

maternal system, such as a female lactating at the time of becoming pregnant a second time. When the stress is removed, the dormant embryos may be reactivated and continue in their development. Lactation-induced diapause is common in rodent species. Rodents reestablish estrus behavior almost immediately following parturition, which may result in the animals becoming pregnant while still nursing young. These developing embryos then become dormant until the previous offspring are weaned (Renfree and Shaw, 2000). Some marsupials may exhibit a combination of obligatory and facultative diapause, where embryonic developmental delays brought about by lactation are further exacerbated by photoperiodic signaling.

Mice that have previously given birth will immediately exhibit estrous behaviors and are receptive to mating (Marois, 1982). If fertilized, the resulting embryos will develop until the blastocyst stage. Progesterone secretions from the corpus luteum are essential for normal development to the blastocyst stage (Zhang and Murphy, 2014). During this period, if metabolic stresses are present, the embryo will enter into a state of decreased metabolic activity. The embryos slowly hatch from the zona pellucida and continue to develop until the blastocyst size has increased to nearly 130 cells, remaining roughly this size for the duration of diapause (McLaren, 1968). During diapause, embryos will be spaced and positioned ready for implantation with apposition occurring, however, implantation does not take place at this time (Nilsson, 1974). Little is known about the mechanism by which embryos are able to decrease proliferation and metabolic activity, but a current theory in mice involves the over production of 38 MicroRNA (miRNA) molecules, some of which target genes related to implantation and cell proliferation (Gurtan et al., 2013; Liu et al., 2012). These miRNAs bind to target mRNA

and lead to destabilization and/or inhibition or translation, which serves to decrease expression of the mRNA. By inhibiting these key target genes the embryo is unable to implant and proliferate.

Upon receiving signals from the uterus, the embryo becomes reactivated and within 4-16 h DNA and protein synthesis, along with cell proliferation are increased, followed shortly by implantation (Fenelon et al., 2014). In rats, the estradiol surge that is secreted during late morning of the fourth day following mating, has been shown to induce implantation (McCormack and Greenwald, 1974). Paria et al. (1990) demonstrated that mice blastocysts incubated *in vitro* with the catechol estrogen 4-OH-estradiol would implant when replaced in the uterus. These results seem to suggest that estradiol and its metabolite 4-OH-estradiol could be maternal signals that reactivate rodent embryos to terminate diapause.

The Mink and Obligatory Embryonic Diapause

Obligate embryonic diapause has been studied in some detail in the Western spotted skunk and the American mink (Aitken, 1980; Mead, 1981; Murphy, 2012). Following mating in early March, mink eggs are fertilized in the oviducts and/or bursa surrounding the ovary and then develop to the blastocyst stage by the eighth day *post coitum* as they enter the uterine lumen (Sundqvist et al., 1989). At this time they enter a dormant state (diapause) until being reactivated during late March. Implantation sites are usually detected during the first week of April. Pregnancy, which is defined as the duration of time from implantation to parturition is approximately 30-31 days (Enders,

1952). The duration of diapause can be quite variable from only a few days to as many as 60, depending upon when the female was last mated.

After ovulation the ruptured follicle transforms into a corpus luteum and begins secreting low concentrations progesterone (P₄; Mead, 1993). During this period, luteal cells have only partially differentiated and maintain the ability to divide (Douglas et al., 1998). It is only later, usually after the second mating that circulating P₄ concentrations increase significantly, during late March, contributing to the induction of implantation. The details of implantation in mink are still poorly understood, as exogenous P₄ and/or E₂ will not induce early implantation.

Unlike rodents, which exhibit facultative delayed implantation, mink embryos do not hatch from zona pellucida until after the blastocyst is reactivated (Fenelon et al., 2014). Subsequent to reactivation, blastocyst cell division, differentiation, and protein synthesis increase dramatically (Desmarais et al., 2004).

In mink the trigger for the termination of embryonic diapause is the increasing photoperiod during and after the vernal equinox. As the daily duration of melatonin secretion decreases, prolactin (PRL) secretion appear to increase, which is considered by many to be the signal which terminates embryonic diapause in mink (Murphy et al., 1990; Fenelon et al., 2014). Murphy et al. (1979) determined in ferrets, a close relative to the mink, that hypophysectomy terminated luteal function. This was not surprising as the pituitary produced many gonadotrophic factors (FSH, LH and PRL). However, luteal function was restored following prolactin treatment. This experiment was later repeated in mink (Murphy et al., 1981) and prolactin treatment was sufficient to restore luteal

function. In the same study, early termination of diapause in mink was also induced experimentally by prolactin injections. Reactivation is initially visible three days following prolactin administration with embryos implanting about 13 days after prolactin treatment (Desmarais et al., 2004). In mink, prolactin secretions reactivates dormant luteal cells (Murphy, 1979) and stimulates increased progesterone secretions (Murphy et al., 1993). As luteal cells become activated, prolactin receptors are up-regulated and further stimulation results in the production of increased progesterone synthesizing enzymes (Douglas et al., 1998).

Uterine Control of Blastocyst Dormancy and Reactivation

The termination of embryonic diapause and induction of implantation appears to be predominantly under uterine control. Chang (1968) transplanted diapausing mink blastocysts into the uteri of ferrets, which do not exhibit diapause, and observed the immediate reactivation of the dormant mink embryos. Conversely, when ferret blastocysts were transplanted into mink uteri, a state of dormancy occurred. This evidence indicates that the signal/s regulating diapause and its termination emanate from the uterus and are not intrinsic to the embryo.

The uterine factor/s responsible for reactivating the mink blastocyst are unknown. Potential uterine signaling molecules include the polyamines spermine, spermidine and putrescine (Lefèvre and Murphy, 2009; Lefèvre et al., 2011a, 2011b, 2011c). As evidence, when mink were treated with prolactin (known to induce implantation) uterine concentrations of polyamines increased two-fold when compared to controls. Moreover, blastocyst reactivation *in vitro* and *in vivo* has been achieved with exogenous polyamines.

Conversely, mink treated with inhibitors of polyamine synthesis had inhibited blastocyst reactivation, resulting in active embryos becoming dormant.

The Physiology of Implantation

Some aspects of the physiology of implantation in mink are similar to other mammals. The secretion of E₂ during follicular development promotes uterine proliferation and differentiation. After ovulation, P₄ stimulates uterine glandular development and secretion of a nutrient rich material called histotroph. Collectively the actions of E₂ and P₄ prepare the uterus for implantation, which can only occur during a narrow window of time called the “window of implantation”. In addition to E₂ and P₄, uterine produced cytokines, growth factors and hormones regulate the window of implantation (Bazer et al., 2010).

During implantation, the re-activated blastocyst hatches from the thick glycoprotein coat that surrounds the embryo called the zona pellucida (ZP). The ZP is formed prior to ovulation, as the oocyte is developing, from secretions from the follicle and the oocyte itself (Wassarman and Litscher, 2012). After ovulation as the egg enters the oviduct on its way toward the uterus. The ZP is a meshwork of proteins that is quite porous, rendering it permeable to large macromolecules (nutrient, hormones and growth factors) and even many small viruses (Keefe et al., 1997). The ZP is responsible for sperm recognition, attachment and also assists in the prevention of polyspermic fertilization (Wassarman, 1999). Following fertilization the ZP protects the embryo as it continues its progress toward the uterus. During implantation, the blastocyst and uterus

produce lysins and other enzymes that promote the digestion of protein filaments, permitting the blastocyst to emerge (hatch) from the ZP (Das and Holzer, 2012).

After the shedding of the ZP, attachment of the embryo to the uterine epithelium can only take place if the glycocalyx covering the epithelial cells is digested and specific adhesion molecules are produced by the uterus (Meseguer et al., 2001; Wang and Dey, 2006). Following attachment, proteases released by the embryo initiate digestion of the underlying endometrium along with signaling-molecule-induced apoptosis (Carson et al., 2000). Upon digestion, the embryonic trophoblast cells invade the uterine endometrium and begins the formation of the endotheliochorial placenta (Carson et al., 2000; Pfarrer et al., 1999).

Pre-embryonic Nutrient Requirements

Pre-embryos require lactate, pyruvate and glucose to develop to the blastocyst stage (Brown and Whittingham, 1991; Burton et al., 2002; Pantaleon et al., 2008). As embryos progress from the 1-cell stage to the morula stage metabolizing lactate and pyruvic acid mainly satisfies their energy demands. As evidence, Brown and Whittingham (1991) demonstrated that incubating 1- cell embryos in media containing lactate and pyruvate was sufficient for 94% of embryos to reach the blastocyst stage, while media containing lactate only as the energy source resulted in only 1% of embryos entering the blastocyst stage. The researchers later demonstrated that using media containing lactate, pyruvate and glucose resulted in 100% of embryos entering the blastocyst stage.

In mice, as the morula continues to grow and divide it begins to utilize glucose in higher quantities (Martin and Leese, 1995; Pantaleon et al., 2008). Glucose use by pig blastocysts increases 3-5 fold when compared to morulas (Swain et al., 2002). Despite using glucose as its major energy source, it appears that embryos (at least until the blastocyst stage) do not undergo cellular respiration to produce ATP, but instead use pathways similar to that of cancer cells, known as the Warburg effect, or aerobic glycolysis, in which pyruvate is metabolized to lactate instead of entering the TCA cycle (Krisher and Prather, 2012). Typically glucose is metabolized through glycolysis to produce ATP and pyruvate, which is then converted through the steps of the tricarboxylic acid (TCA) cycle. The products are then oxidized in the electron transport chain to produce relatively large amounts of ATP (36 per molecule of glucose). Early embryos appear to use only glycolysis, producing only two molecules of ATP per molecule of glucose. Krisher and Prather (2012) hypothesize that during this time of rapid cell division shunting all glucose toward ATP production may be counterproductive, suggesting instead that more glucose is shifted to biosynthetic pathways such as the pentose phosphate pathway. This would enable the production of nucleic acids and allowing carbon sources to be utilized by the rapidly dividing cells, instead of being lost as carbon dioxide.

Glucose appears to be essential for blastocyst formation, yet uterine and oviductal fluid glucose concentrations are lower than plasma glucose concentrations. During the first trimester of pregnancy in women, fasting plasma glucose concentrations were 4.3 mmol/L, a slight decrease when compared to non-pregnant women (4.5 mmol/L; Riskin-

Mashiah et al., 2011). Glucose concentrations in pregnant women's oviductal and uterine fluids were 0.5 mmol/L and 3.15 mmol/L respectively (Gardner et al., 1996).

Nevertheless, numerous investigators have confirmed the reliance of embryos on glucose for development (Martin and Leese, 1995; Pantaleon et al., 2008). Conversely, high uterine glucose concentrations (diabetic mothers) have been shown to be detrimental to embryo survival (Lea et al., 1996; Moley et al., 1991). When embryos were incubated in media containing 10x normal uterine glucose concentrations, embryonic cells underwent apoptosis and the embryos did not survive (Moley et al., 1998). It is apparent that uterine glucose concentrations must be properly regulated to maintain an adequate source of energy for the developing embryo while preventing embryotoxic overexposure. It would seem reasonable to propose that glucose concentrations in uterine fluid (histotroph) would be regulated.

Early exposure to glucose may also be required for embryo survival. Apart from its roles as an energy source for the embryo, early glucose exposure appears to be necessary in the hatching of the mouse embryo from the zona pellucida (Wordinger and Brinster, 1976). Without hatching, embryos could not implant and are lost (Seshagiri et al., 2009). It is possible that inadequate glucose at a critical stage of development could contribute to embryonic loss. Interestingly, several genes thought to be involved in the digestion of the zona pellucida were up regulated in developing trophectoderm cells as the embryos utilize increasingly more glucose (Ozawa et al., 2012). It is therefore possible that glucose could be employed as a signaling molecule to induce the production of these digestive enzymes.

Potential Significance of Uterine Histotroph to Reproductive Success

Histotrophic secretions are produced by the uterine endometrial glands of all mammals thus far investigated and are rich in proteins, fats, carbohydrates, growth factors, ions and hormones (Bazer, 1975; Burton et al., 2001, 2002; Wimsatt, 1950). Before the formation of the placenta is complete, blastocysts are reliant upon histotroph for survival, development and perhaps implantation (Spencer et al., 2004). As evidence, ewes treated with a synthetic progestin for eight weeks following birth, resulted in uteri that were deficient in glands (Gray et al., 2001a). Upon fertilization embryos exhibited normal development to the blastocyst stage (day 9 after mating). However development beyond this stage was severely inhibited, and most embryos failed to implant and degenerated.

Failure of the embryo to implant in the uterine endometrium is responsible for most pregnancy losses. Implantation failure has been estimated to be responsible for 70% of the pregnancy losses in cattle (Diskin and Morris, 2008), 60% in horses (Morris and Allen, 2002), and 75% in humans (Norwitz et al., 2001). Thus, a better understanding of the physiological mechanisms controlling embryo implantation may lead to improved animal production, cures for human infertility, and perhaps more effective forms of contraception. Because glucose is such a critical nutrient, contributing to pre-embryonic development, it would not seem unreasonable to hypothesize that insufficient glucose in histotroph secretions might result in early pre-embryonic death, and/or failure of embryos to undergo implantation. While the uterus cannot carry out gluconeogenesis, the organ does take up glucose, store it as glycogen and transport glucose from the maternal circulation to the uterus for local utilization by uterus and embryos.

Regulation of Uterine Glycogen Metabolism by Estradiol and Progesterone

Uterine glycogen represents a carbohydrate store that may be catabolized to glucose for secretion in histotroph, secreted as whole glycogen granules (Burton et al., 2002), or used by the uterus as an energy source (Demers et al., 1972). The uterus synthesizes glycogen in a reproductive cycle-dependent pattern. In humans, uterine glycogen concentrations peak during the secretory phase (Milwidsky et al., 1980; Souda et al., 1985), coinciding with the anticipated window of implantation. Decreased uterine glycogen has also been demonstrated in a subset of infertile women (Hughes et al., 1969; Maeyama et al., 1977; Zondek and Stein, 1940). In Rats glycogen concentrations are highest during proestrus and decline until implantation (Demers et al., 1972). Uterine glycogen metabolism has been demonstrated in many species to be regulated by estradiol 17-beta (E_2) and progesterone (P_4).

Estrogen has been shown to increase uterine weight, uterine glycogen accumulation and uterine glycogen synthase activity in mice, rats, rabbits, hamsters and guinea pigs (Bitman and Cecil, 1967; Bitman et al., 1965; Demers and Jacobs, 1973; Demers et al., 1973b; Gregoire et al., 1967; Hall and Khaligh, 1968; Tripathi, 1983). During the estrous cycle of the rat, uterine glycogen concentrations peak at proestrus and estrus as circulating estrogen concentrations are high (Boettiger, 1946; Demers et al., 1972).

Ovariectomized rats, treated with a single dose of estradiol experienced a maximal increase of uterine glycogen 24 h post treatment after which glycogen content slowly diminished. Uterine glycogen content of treated rats at 24 h was elevated 2-5 fold

above control animals (Bitman et al., 1965; Bo et al., 1971; Demers et al., 1972). Whole cross sections of uterine tissue from rats, rabbits, and guinea pigs demonstrated increased glycogen synthase activity which coincided with uterine glycogen accumulation (Demers et al., 1973b).

Progesterone induces species-specific uterine glycogeneolytic and glycogenic actions. The majority of research in rats indicates that progesterone has a mobilizing effect on uterine glycogen (Bo and Atkinson, 1952; Bo et al., 1971; Demers and Jacobs, 1973; Garrison et al., 1973; Poteat and Bo, 1977). In these noted studies, estrogen is used to stimulate uterine glycogen and then progesterone treatment, either alone or with continued estrogen treatment. This results in a decrease of uterine glycogen when compared to estrogen treatment alone. Progesterone also appears to mobilize uterine glycogen in mice (Hall, 1965). Conversely, progesterone stimulates uterine glycogen synthesis in cats (Boomsma and Verhagem, 1982; Boomsma et al., 1982; Jaffe et al., 1985) and human women. These differences appear to be species specific.

In women, uterine glycogen appears to be synthesized by the actions of progesterone. Glycogen in females is also highest during the secretory periods (when progesterone concentrations are high) and lowest during the proliferative phase (when estrogen concentrations are high; Fukuma et al., 1983). As further evidence, postmenopausal women taking estradiol hormone replacement with varying doses of progesterone demonstrated endometrial glycogen accumulation in 91% of patients (Lane et al., 1983). Moreover, women treated with synthetic progestins have shown increased glycogen accumulation in both normal endometrial and in carcinomas of endometrial

tissue. (Fukuma et al., 1983). These results suggest that in women, progesterone promotes glycogenesis in the uterus, but these effects may require the actions of estrogen.

In other species the role of progesterone on uterine glycogen is unclear. In rabbits it has been reported that treatment with estrogen followed by progesterone decreases uterine glycogen (Demers et al., 1973b). However, rabbits primed with estrogen and then given a single dose of progesterone exhibited uterine glycogen accumulation, particularly in the glandular cells (Secchi et al., 1987). This was a rapid and transitory phenomenon that was observed as early as one hour after the progesterone treatment, with estrogen failing to elicit a glycogenic response. In guinea pigs, Demers et al. (1973b) reported a decrease in uterine glycogen to estradiol priming followed by progesterone stimulation. In a subsequent study, Demers et al. (1977) primed guinea pigs with estradiol for two days followed by progesterone treatment for two day. The guinea pigs demonstrated a 100% increase of uterine glycogen, challenging his earlier findings in guinea pigs. Gregoire et al. (1972) reported an increase in guinea pigs uterine glycogen with concomitant estradiol and progesterone treatment when compared to estradiol treatment alone.

The mink uterus synthesizes glycogen in response to estrogen (Rose et al., 2011). Ovariectomized mink treated with estradiol exhibited uterine glycogen concentrations roughly 10-fold higher than controls. Glycogen content of the mink secretory epithelia (glandular and luminal) is highest during estrus, and declines during diapause and pregnancy (Dean et al., 2014). During estrus when circulating E₂ concentrations are high, the mink uterus accumulates glycogen reserves (Figure 1). Following mating and ovulation, mink embryos enter diapause. During this period, mink uterine glycogen has

been reduced compared to estrus concentrations, being mobilized for use by the embryo or the uterus itself. Following implantation, mink uterine glycogen is almost undetectable. These data demonstrate to us a decrease in mink uterine glycogen from estrus to the beginnings of pregnancy. It is possible that mink uterine glycogen is mobilized in response to progesterone that is being secreted by the CL during this period of the reproductive cycle.

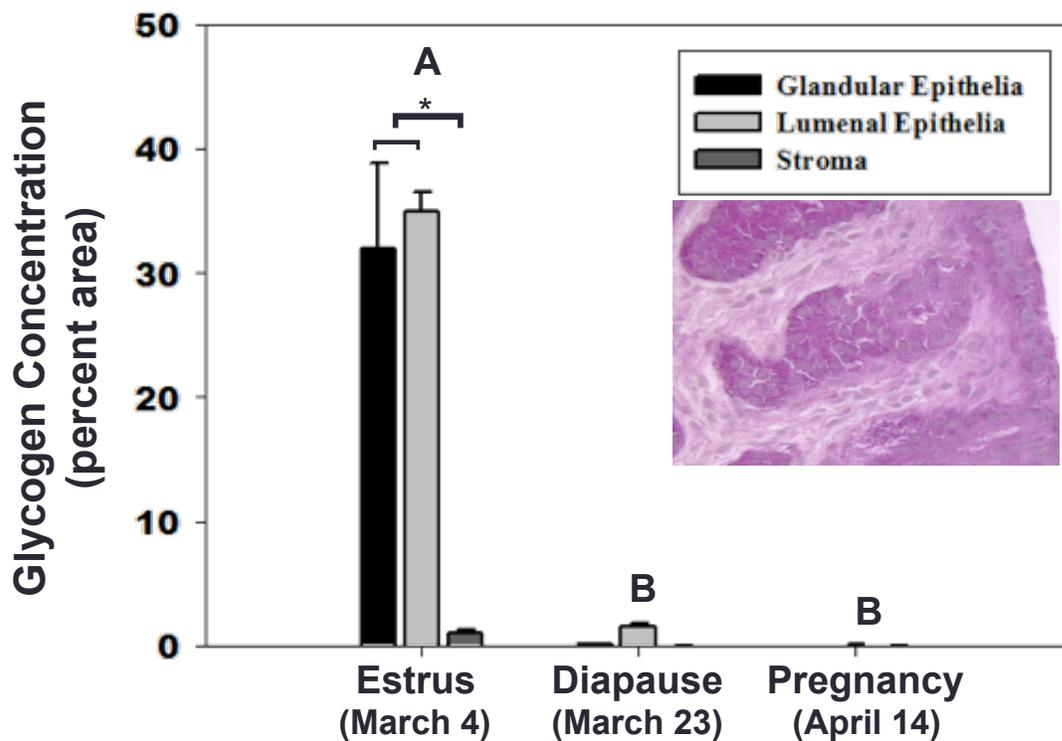


Figure 1. Mean (\pm S.E.) mink uterine glycogen concentrations in the stroma, glandular and luminal epithelia during estrus, embryonic diapause and pregnancy. Differences between samplings were indicated with a different letter. Differences between cell types are indicated by an * (from Dean et al., 2014).

Change in circulating concentrations of E2 and P4 in the mink

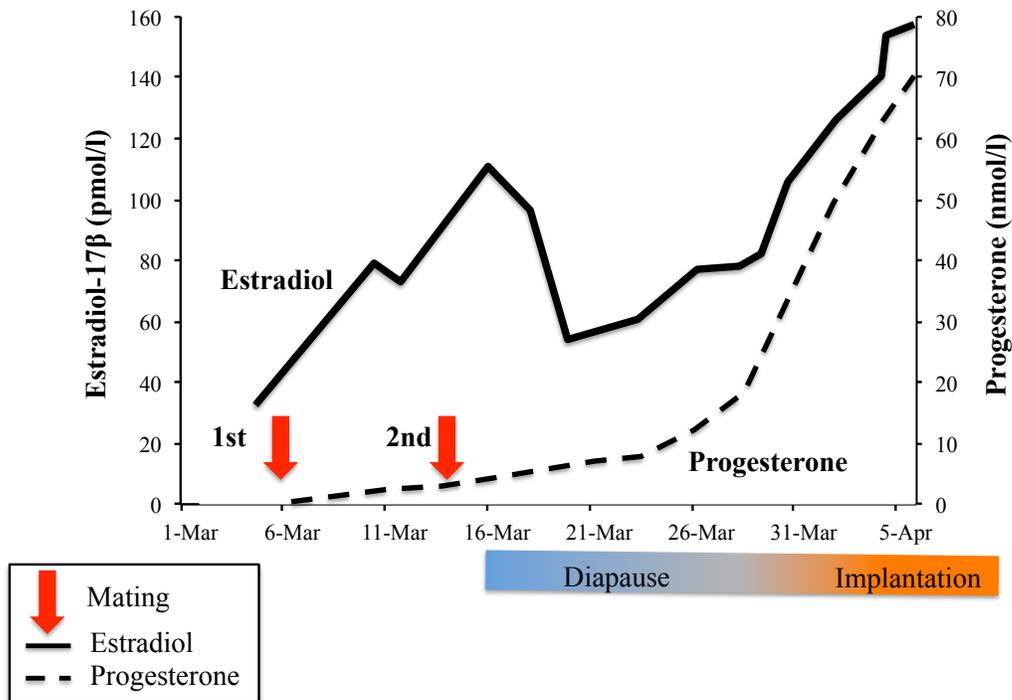


Figure 2. Serum progesterone and estradiol from pregnant female mink during estrous, diapause and pregnancy. Mink were mated twice (red arrows) according to standard ranch practices. (modified from Lagerkvist et al., 1992).

As mentioned previously, mink are seasonal breeders. Several weeks before proestrus and estrus, circulating estradiol concentrations begin to increase (Pilbeam et al., 1979). Estradiol stimulates receptivity to mating and induces uterine changes that prepare the uterus for implantation. Progesterone concentrations begin increasing at the time of the first mating and slowly increase until the vernal equinox. Estradiol concentrations transiently rise until mid-March and then decrease as progesterone production increases (Lagerkvist et al., 1992; Figure 2). During diapause mink CLs enter a less active state, decreasing in size and producing only small amounts of progesterone (Douglas et al.,

1998; Lagerkvist et al., 1992). Following the equinox, the CL become reactivated, following LH and prolactin stimulation (Murphy et al., 1983) and begins producing large amounts of progesterone. Progesterone concentrations peak mid-pregnancy and decrease until parturition (Moller, 1973). After the equinox, estradiol production increases for a second time (Figure 2), possible aiding in implantation.

Exogenous estradiol stimulates glycogenesis in the mink uterus (Rose et al., 2011) and subsequent uterine glycogen concentrations are highest during estrus (Dean et al., 2014). Mink uterine glycogen concentration are mobilized during preimplantation as the CLs are producing modest amounts of progesterone (Dean et al., 2014; Lagerkvist et al., 1992). Progesterone has been implicated in the mobilization of uterine glycogen in rats and mice (Hall, 1965; Poteat and Bo, 1977). It is therefore possible that the mink uterus is mobilizing glycogen in response to progesterone stimulation.

To determine if P₄ might increase glycogenolysis and/or decrease glycogenesis in the mink uterus we designed an experiment to test the effects of E₂ and P₄ alone or E₂ followed by P₄ (E₂ → P₄) to simulate E₂ priming. Uteri were analyzed for, (1). Glycogen content of endometrium, myometrium, stroma, glandular and luminal epithelia, (2). Relative mRNA expression levels for glycogen synthase 1 (*Gys1*), glycogen phosphorylase (*Pygm*), hexokinase 1 (*Hk1*), and glucose-6-phosphatase 3 (*G6pc3*), and (3). Protein levels for Gys1 and Hk1.

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Estradiol stimulates glycogen synthesis whereas progesterone promotes glycogen catabolism in the uterus of the American mink (*Neovison vison*)

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ABSTRACT: Glycogen synthesis by mink uterine glandular and luminal epithelia (GE and LE) is stimulated by estradiol (E₂) during estrus. Subsequently, the glycogen deposits are mobilized to near completion to meet the energy requirements of pre-embryonic development and implantation by as yet undetermined mechanisms. We hypothesize that progesterone was responsible for catabolism of uterine glycogen reserves as one of its actions to ensure reproductive success. Mink were treated with E₂, P₄ or vehicle (controls) for three days and uteri collected 24 h (E₂, P₄ and vehicle) and 96 h (E₂) later. To evaluate E₂ priming, mink were treated with E₂ for three days, then P₄ for an additional three days (E₂→P₄) and uteri collected 24 h later. Percent glycogen content of the uterine epithelia was greater E₂ + 96 h (GE = 5.71 ± 0.55; LE = 11.54 ± 2.32) than E₂ + 24 h (GE = 3.63 ± 0.71; LE = 2.82 ± 1.03) and both were higher than controls (GE = 0.27 ± 0.15; LE = 0.54 ± 0.30; P ≤ 0.05). Treatment as E₂→P₄ reduced glycogen content (GE = 0.61 ± 0.16; LE = 0.51 ± 0.13), to levels not different from controls, while concomitantly increasing catabolic enzyme (glycogen phosphorylase and glucose-6-

phosphatase) gene expression and amount of phospho-glycogen synthase protein (inactive) in uterine homogenates. Interestingly, $E_2 \rightarrow P_4$ increased glycogen synthase 1 mRNA and hexokinase 1 mRNA (and protein). Our findings suggest to us that while E_2 promotes glycogen accumulation by the mink uterus during estrus and pregnancy, it is P_4 that induces uterine glycogen catabolism, releasing the glucose that is essential to support pre-embryonic survival and implantation.

Key Words: Progesterone, estradiol, glycogen, uterus, mink

Running Title: Mink uterine glycogen metabolism

INTRODUCTION

Pre-embryonic growth, implantation and early fetal development are dependent upon uterine glandular and luminal epithelial cell secretions (histotroph) containing glucose, glycogen, amino acids, fats, ions and hormones (Burton *et al.* 2002; Gray *et al.* 2001; Hempstock *et al.* 2004). Although gluconeogenesis does not occur in the uterus (Yáñez *et al.* 2003; Zimmer and Magnuson 1990) glucose is taken up, transported into the uterine lumen, metabolized as a fuel source or when in excess, stored as glycogen.

Glycogen synthesis begins with phosphorylation of glucose by hexokinase (Hk), producing glucose-6-phosphate, which is isomerized to glucose-1-phosphate and converted to uridine diphosphate glucose (Ferrer *et al.* 2003). Glucosyl units from the latter are transferred to non-reducing ends of glycogen molecules by glycogen synthase (Gys). Glycogen is catabolized by glycogen phosphorylase (Pyg), releasing glucose-1-phosphate, which may be isomerized to glucose-6-phosphate and enter the glycolytic pathway, or be dephosphorylated by glucose-6-phosphatase (G6pc), releasing glucose for export to the systemic circulation and/or uterine lumen.

Uterine glycogen content in rodents peaks during proestrus to estrus, and is mobilized during implantation and pregnancy (Demers *et al.* 1972; Greenstreet and Fotherby 1973a). Similarly, the human uterus stores large amounts of glycogen during the late proliferative to early secretory phases, that is mobilized by the late secretory phase (Cornillie *et al.* 1985; Spornitz 1992; Verma 1983). In mink, uterine glandular and luminal epithelial glycogen content decreased 99% between estrus and the peri-implantation period (Dean *et al.* 2014). Mink are seasonal breeders exhibiting obligatory embryonic diapause and may have blastocysts (as many as 17) in a state of arrested development for 50-60 days *post coitum*, resulting in delayed implantation (Enders 1952; Hansson 1947). It is likely that mink uterine glycogen reserves are a potential source of energy that supports pre-embryonic survival and implantation. As evidence, the uterine glycogen content of sterile women or those exhibiting spontaneous abortions, was greatly diminished when compared to normal women and in some cases absent (Girish *et al.*, 2012; Hughes *et al.*, 1963, 1969; Maeyama *et al.*, 1977; Rubulis *et al.*, 1965; Zawar *et al.*, 2003).

Uterine glycogen synthesis is stimulated by estradiol (E_2) in rats, rabbits, guinea pigs (Demers *et al.* 1973a,b; Demers *et al.* 1972; Greenstreet and Fotherby, 1973b) and mink (Rose *et al.* 2011). In the ovariectomized rat, exogenous E_2 increased uterine glycogen content as expected (Paul and Dutttagupta 1973). However, when treated concomitantly with E_2 and progesterone (P_4), uterine glycogen concentrations were reduced when compared to E_2 alone. Demers and Jacobs (1973) treated ovariectomized rabbits and guinea pigs with E_2 followed by P_4 ($E_2 \rightarrow P_4$) to simulate E_2 priming and reported a reduction in uterine glycogen content and a 2-fold increase in glycogen

phosphorylase activity, compared to E₂ alone. These findings suggest that P₄ promotes uterine glycogen catabolism and/or antagonizes the glycogenic actions of E₂.

Circulating E₂ concentrations in mink increase after mating, then decline during the variable period of embryonic diapause and increase again during the peri-implantation period (Lagerkvist *et al.* 1992). Plasma P₄ concentrations in mink remain low until the vernal equinox (regardless of the time of mating), then increase concomitantly with E₂ during the peri-implantation period. It is possible that E₂ promotes glycogen accumulation during proestrus and estrus, and that after mating and ovulation, P₄ triggers glycogen mobilization. If this is true, the mink uterus must be extremely sensitive to P₄ at this time, since circulating concentrations of the steroid, while increasing, are very low until late diapause (Lagerkvist *et al.* 1992). Nevertheless, increased uterine sensitivity to P₄, in response to E₂ induced up regulation of P₄ receptors, may result in a uterus that responds to low (perhaps unchanging) P₄ concentrations.

To determine if P₄ might glycogen catabolism and/or decrease synthesis in the mink uterus, animals were bilaterally ovariectomized and treated with E₂ and P₄ alone or to simulate E₂ priming, E₂ followed by P₄ (E₂→P₄). Uteri were analyzed for; (i) glycogen content of endometrium, myometrium, stroma, glandular and luminal epithelia; (ii) relative messenger RNA (mRNA) expression levels for glycogen synthase 1 (*Gys1*), glycogen phosphorylase (*Pygm*), hexokinase 1 (*Hk1*) and glucose-6-phosphatase 3 (*G6pc3*); and (iii) protein levels for *Gys1* and *Hk1*.

MATERIALS AND METHODS

Animals

Twenty-five adult (18-19 months old) dark, preparturient female mink (6-8 offspring per litter), were moved from outdoor ranch conditions to the indoor animal facility at Idaho State University (ISU) in early November. Mink were housed individually, fed a mixture of chicken and fish by-products daily and water *ad libitum*. Animals were exposed to a daily photoperiod approximating natural photoperiodic changes for southeastern Idaho (Rose 1995) at room temperatures between 22-28°C. All procedures involving animal care, surgery and hormone treatments were approved by the Institutional Animal Care and Use Committee of ISU, and conformed to the Guide for the Care and Use of Laboratory Animals (Protocol #6410909).

Hormones

Estradiol 17 beta (E_2 ; R187933; Sigma Chemical Co., St. Louis, MO, USA), and progesterone (P_4 ; P0130; Sigma Chemical Co.) were initially dissolved in 95% ethyl alcohol (EtOH) and then mixed in sesame seed oil as a vehicle for subcutaneous injection.

Treatments

On Day 0 (November 17) all mink were bilaterally ovariectomized through a single mid-ventral incision while under ketamine hydrochloride anesthesia (50 mg/kg body weight; Fort Dodge Animal Health, Fort Dodge, IA, USA). Animals were subsequently returned to their cages for 11 days, to allow for recovery and natural elimination of residual ovarian hormones. On Day 12, animals were assigned at random to one of five groups (Fig. 1; $n = 5/\text{group}$). Mink in groups 2, 3, and 4 were each injected once daily on days 12, 13 and 14 with E_2 (50 $\mu\text{g}/\text{kg}$ body weight). Mink in group 5 each received daily injections of P_4 (25 mg/kg body weight) on days 12, 13 and 14 while mink

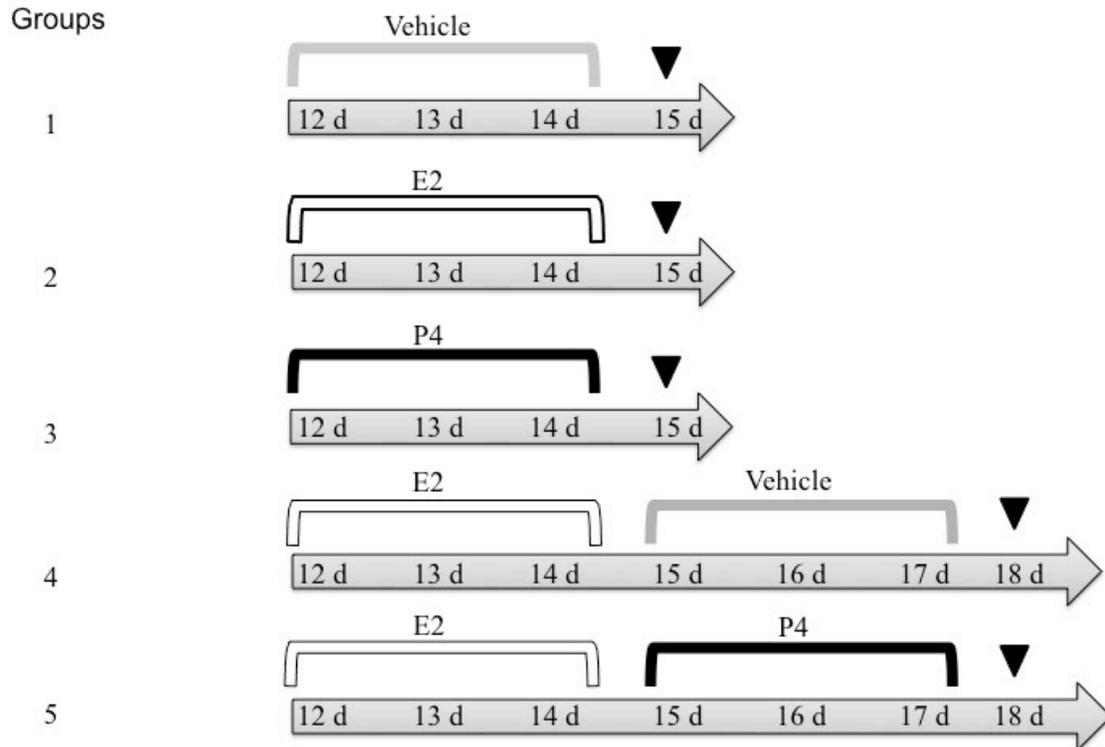


Fig. 1 Twenty-five adult female mink were bilaterally ovariectomized on November 17 (Day 0). On Day 12, mink were assigned at random to one of five groups (N = 5/group) and treatments initiated. Mink in groups 2, 3 and 4 were each injected once daily on days 12, 13 and 14 with estradiol 17beta (E₂; 50 µg/kg body weight). On the same days, mink in group 5 each received daily injections of progesterone (P₄; 25 mg/kg body weight). Mink in group 3 received daily injections of vehicle on days 15, 16 and 17 while mink in group 4 received daily injections of P₄ on the same days to simulate E₂ priming. Mink in group 1 received vehicle only on days 12, 13 and 14 and represented controls. All animals were sacrificed 24 h after the last perturbation (▼) with a lethal dose of Sleep-A-Way (Fort Dodge Animal Health, FT. Dodge, IA, USA), the uteri collected and quick frozen in liquid nitrogen. d, days

in group 4 received P₄ injections on days 15, 16 and 17. Animals in group 3 each received daily injections of vehicle on days 15, 16 and 17 while mink in group 1, received injections of vehicle only on days 12, 13 and 14 and represented controls. Twenty-four hours after the last perturbation, animals were killed with a lethal dose of Sleep-A-Way (Fort Dodge Animal Health), uteri were collected and quick frozen in liquid nitrogen.

Glycogen Concentrations in Uterine Homogenates

Glycogen concentrations were determined using a modified procedure of Good *et al.* (1933). Uterine tissue (70-120 mg) from each mink was lyophilized for 3 days and homogenized in 20 volumes of 30% KOH. A 125 μ L aliquot of the homogenate was incubated at 100°C for 30 min to denature enzymes and destroy free glucose. To isolate glycogen, samples were diluted with 1.2 vol (150 μ L) 95% EtOH, frozen at -80°C for 60 min, thawed and centrifuged at 9,600 \times g for 10 min. After discarding the supernatant, tubes were inverted and the pellet dried overnight. Subsequently, 100 μ L of 1.0 N HCL was added to the pellet, heated at 100°C for 2.5 h to break down glycogen to D-glucose. Glucose concentrations, as an indicator of glycogen content, were determined using Wako Glucose Auto Kit (439-90901; Wako Chemicals USA, Inc., Richmond, VA, USA) according to the manufacturer's instructions. Glucose was quantified spectrophotometrically ($\lambda = 505$ nm) by comparing unknowns against a standard curve of increasing glucose concentrations. All samples were analyzed in duplicate, in a single assay with an intra-assay coefficient of variation of 2.0%.

Localization of glycogen deposits in uterine cross-sections

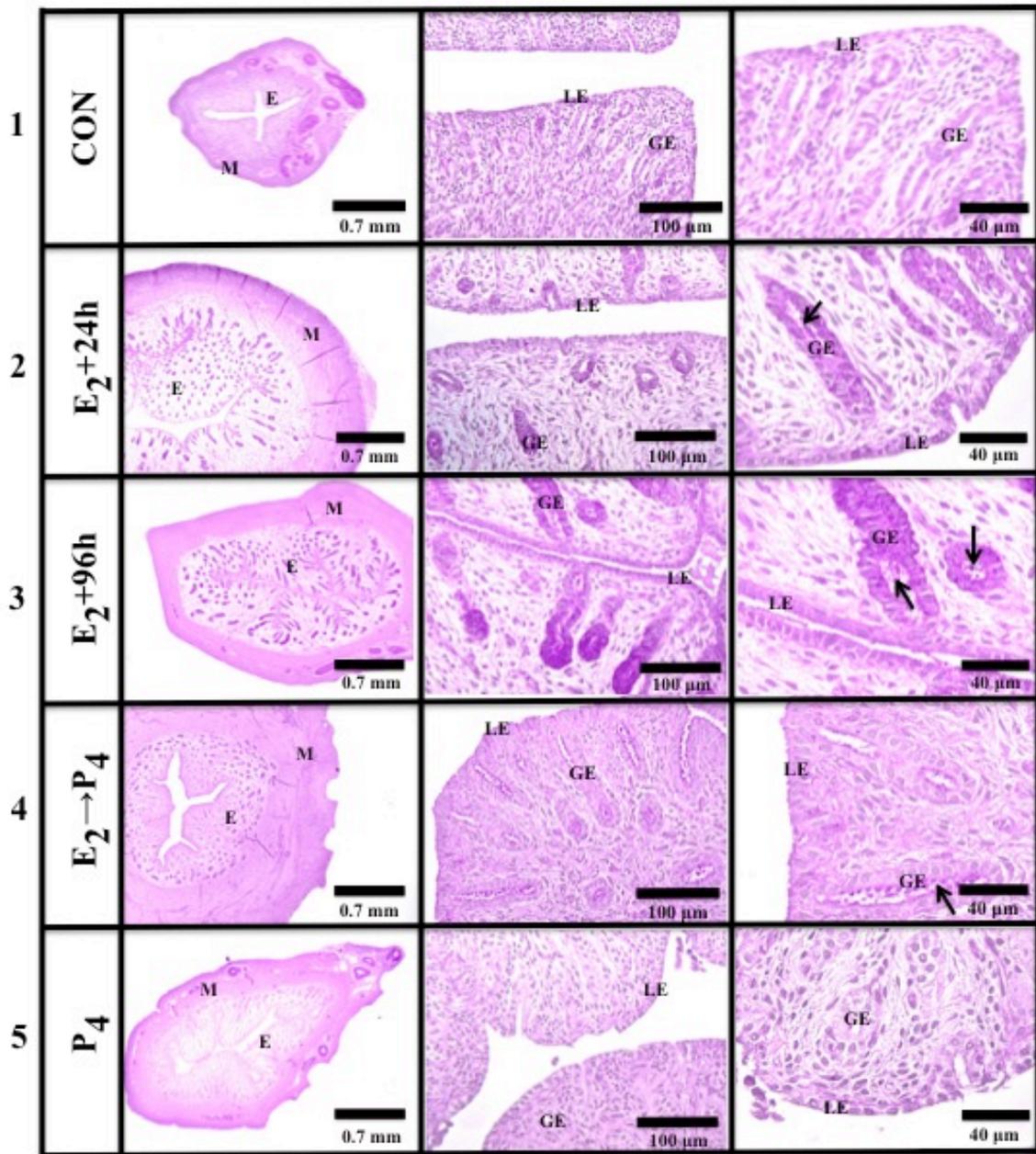


Fig. 2. Mink uterine cross-sectional images. Animals were injected with E_2 for three days and sacrificed 24 h ($E_2 + 24$ h) and 96 h ($E_2 + 96$ h) after the last injection. To simulate E_2 priming, mink were treated for three days with E_2 and then an additional three days with P_4 and sacrificed 24 h later ($E_2 \rightarrow P_4$). Mink were also treated with P_4 for three days and sacrificed 24 h later ($P_4 + 24$ h). Controls (CON) received injections of vehicle only for three days and were sacrificed 24 h later. All sections (7 μ m) were stained with periodic acid-Schiff (PAS) and counterstained with hematoxylin. Images were captured at 25 \times (left column), 200 \times (middle column) and 400 \times (right column). Arrows identify areas of positive PAS staining within the glandular lumens. M, myometrium; E, endometrium; GE, glandular epithelium; LE, luminal epithelium.

Uterine samples were fixed in 10% neutral buffered formalin, dehydrated and mounted in paraffin blocks. Transverse uterine sections (7 μm) were incubated with periodic acid-Schiff (PAS) reagent and counter stained with hematoxylin. Images were captured digitally at 25, 200 and 400 \times and analyzed using ImageJ software (Abramoff *et al.*, 2004). For quantification of endometrial and myometrial glycogen content, three consecutive uterine cross-sectional images from each mink were analyzed at 25 \times (whole uterine cross-section) whereas for glandular and luminal epithelial glycogen content, a portion (at 400 \times) of each of three consecutive uterine cross-sections were analyzed. Because PAS stains carbohydrates in addition to glycogen, an additional cross-section was pre-treated with diastase (A8220, Sigma Chemical Co.), to digest glycogen prior to PAS staining and served as a negative control. Glycogen content of each tissue was quantified by subtracting negative control values from sections stained with PAS without diastase and then expressed as a percentage of the area that stained positive for glycogen.

RNA isolation and conversion to cDNA

Total RNA was isolated from 20-30 mg uterine tissue from each mink using Qiagen RNeasy Fibrous Tissue Mini Kit (74104, QIAGEN, Valencia, CA, USA) as previously described (Rose *et al.*, 2011). Samples were screened for protein contamination by measuring light absorption of each sample at 260 nm (DNA and RNA) and 280 nm (protein). Only RNA preparations with a 260/280 ratio of 1.9 or greater were used for quantitative PCR (qPCR) analysis. To eliminate any contaminating DNA, samples were treated with genomic DNA (gDNA) wipeout buffer prior to the reverse-transcription reaction. Conversion of unstable RNA to stable cDNA transcripts (first

strand transcripts) was achieved using QuantiTect Reverse Transcription Kit (205311, QIAGEN) according to the manufacturer's instructions.

Table 1. Primers for qPCR of mink uterine gene transcripts and amplicon characteristics.

Genes	Forward and reverse primers (5' to 3')	Amplicon (bp)	Melt temperature (°C)	Accession No.
<i>Gys1</i>	AAGATGGCAGCCACGGACAAATTC TCAATAGTAGTGCCGACAGGGTCA	110	87.5 ± 0.50	KM084858
<i>Pygm</i>	TCATCACCCCTGTACAACCGCATCA AGCCTTCCCTCCAATCATCACAGT	80	79.0 ± 0.35	KM084856
<i>Hkl</i>	AATGCCAAAGAAATCCTGACCCGC TGGGTGTGCCCTTGTTATCTCGAA	163	84.1 ± 0.43	KM026508
<i>G6pc3</i>	CCCTACTGCCTCGCCCAGGT TGGGCTTGTGAGCTGGCCCTA	117	86.4 ± 0.50	KM015512
<i>Gapdh</i>	AGGTCATCCATGACAACCTTCGGCA ACCAGTGGAAGCAGGGATGATGTT	152	84.8 ± 0.45	KM025344

Gys1, glycogen synthase 1; *Pygm*, glycogen phosphorylase-muscle; *Hkl*, hexokinase 1; *G6pc3*, glucose 6-phosphotase 3; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase;

Primer design

To produce primers specific to mink cDNA, we first used the Basic Local Alignment Search Tool (BLAST) from the National Center of Biotechnology Information (NCBI; NIH <http://www.ncbi.nlm.nih.gov/sites/entrez?dbgenome>), and designed conventional PCR primers for each gene of interest based on regions of homology between rats and other species. Using these primers, we amplified mink cDNA using the Qiagen HotStarTaq® Master Mix Kit (203443, QIAGEN). We separated the resulting cDNA fragments by gel-electrophoresis and the band containing the gene fragment of interest were purified using a Millipore Ultra Free DNA Centrifugal Filter (42600, Millipore Corporation, Billerica, MA, USA). The nucleotide sequence for each gene of interest was then determined using Biosystems 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Based on the resulting mink nucleotide sequence for

each target gene of interest, primers were designed for qPCR (Table 1), and purchased from Integrated DNA Technologies (Coralville, IA, USA).

Quantitative PCR (qPCR)

All qPCR reactions were carried out in triplicate using QuantiTect SYBR® Green PCR Kit (204143, QIAGEN) as per the manufacture's instructions. Forward and reverse primer concentrations were previously optimized and were added to 5 µL cDNA template (100-200 ng) per reaction. The PCR products were detected in real time by measuring SYBR-green fluorescence during the annealing stage using MJ Research Chromo4 Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA). Efficiency of amplicon doubling during each PCR cycle was, *Gapdh*, 97%; *Gys1*, 103%; *Pygm*, 98%; *G6pc3*, 97%; and *Hkl*, 100%. Negative controls contained no template and amplification was never above background. Primer specificity was determined using melt curve analysis, which resulted in a single melting temperature for each amplicon.

Fold changes in gene expression were determined using the relative standard curve method (Pfaffl, 2001). Standard curves were generated from three pooled control mink uteri. The cDNA from these uteri was diluted (1:10, 1:100, 1:1,000 and 1:10,000) or undiluted to construct standard curves (cDNA ng/ml vs. quantification cycle, Cq) for each gene of interest ($R^2 = 0.98-0.99$). Data were averaged by treatment group (n = 5 mink/group, each assayed in triplicate) and expressed in terms of relative fold-difference compared to controls.

We chose *Gapdh* with which to normalize our data, as it showed the least variation in expression (*Gapdh* Cq for E₂ + 24 h = 21.59 ± 0.22, for E₂ + 96 h, Cq = 21.54 ± 0.38, for E₂→P₄, Cq = 21.64 ± 0.55, for P₄ = 21.72 ± 0.33 and for controls, Cq = 22.28 ±

0.22), as compared to *18s rRNA* (Cq for E₂ + 24 h, = 17.47 ± 0.58. for E₂ + 96 h, Cq = 18.34 ± 0.39, for E₂→P₄, Cq = 17.97 ± 0.07, for P₄ = 18.60 ± 0.56 and for controls, Cq = 23.05 ± 0.48).

Western blot analysis

Uterine proteins were isolated using RIPA Lysis Buffer (sc-24948 Santa Cruz Biotechnology, Dallas, TX, USA) according to the manufacturer's instructions. Protein concentrations were determined using Pierce BCA Protein Assay Kit (23225, Thermo Scientific, Rockford, IL, USA). Enzyme proteins of interest were resolved by molecular weight using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and blocked for 1 h in 5% milk buffer containing Tris-buffered saline-Tween 20 (TBS-T: BP337-100; Fisher Scientific, Pittsburgh, PA, USA) to reduce non-specific binding. Membranes were subsequently incubated overnight at 4°C with primary antibodies specific for Gys (3886S, Cell Signaling Technology, Danvers, MA, USA, 1:500 dilution), phosphorylated Gys (pGys: 3891S, Cell Signaling Technology, 1: 500 dilution), Hk1 (2024S, Cell Signaling Technology, 1:1000 dilution). Subsequently the membranes were washed in TBS-T to remove excess primary antibodies. At that time we incubated the membranes with secondary antibody (anti rabbit immunoglobulin G conjugated to horseradish peroxidase; 7074, Cell Signaling Technology, 1:1000 dilution) for 2 h to detect the enzyme proteins of interest. Concomitantly (as a loading control) we incubated membranes with a primary antibody conjugated to horseradish peroxidase against beta-actin (Actb-HRP, 5125S, Cell Signaling Technology, 1:10,000 dilution) for 2 h. To blots with HRP conjugated to the secondary antibody we added Immobilon Western Chemiluminescent HRP Substrate

(WBKLS0100, Millipore Corporation), while samples that were incubated with Actb-HRP were incubated with Novex HRP_Chromogenic Substrate (100002903, Invitrogen, Grand Island, NY, USA). The resulting blots were photographed using Bio-Rad VersaDoc 3000 Imager (BioRad Laboratories). The relative amount of each protein of interest was determined using ImageJ gel analyzer to quantify band density and then normalized to beta-actin as a loading control. Data were then expressed as relative protein levels/ β -actin for each gene of interest. All samples were analyzed in triplicate.

To validate the use of primary antibodies against mink proteins, both mink and rat proteins were concurrently analyzed. For each protein assayed we detected only a single band corresponding to the correct molecular weight of each protein (Gys, pGys and Hk1) in both rat and mink uterine tissue (data not shown). All primary antibodies were produced against human proteins and validated by the manufacturer against rat proteins. Antibodies against Gys were not specific to a single isoform and would therefore bind to both Gys1 (muscle) and Gys2 (liver).

Statistical Analysis

All data were analyzed by one-way analysis of variance, followed by Tukey's *post hoc* test (SigmaPlot, version 12.5, Systat Software Inc., San Jose, CA). Differences were considered significant at $P \leq 0.05$.

RESULTS

Uterine endometrial area was four-fold greater at 24 h and six-fold greater at 96 h after the last E_2 injection when compared to ovariectomized controls (Table 2; $P \leq 0.05$). The endometrial area of mink treated as $E_2 \rightarrow P_4$, was less than mink treated with $E_2 + 96$

h treated animals ($P \leq 0.05$), but did not differ from $E_2 + 24$ h mink. Although the endometrial area of $E_2 \rightarrow P_4$ and $P_4 + 24$ h treated mink tended to be greater than controls, the differences were not significant. Myometrial area was greater at 24 h (3.5 fold) and 96 h (2.5 fold) after the last E_2 injection when compared to controls (Table 2; $P \leq 0.05$). The myometrial area of mink treated as $E_2 \rightarrow P_4$ did not differ from mink treated with E_2 only (24 and 96h) and was greater when compared to controls ($P \leq 0.05$). Myometrial area of mink treated with $P_4 + 24$ h did not differ from ovariectomized controls. Gross uterine glycogen concentrations in mink treated as $E_2 + 24$ h were higher than for all other treatments (Table 3; $P \leq 0.05$). In $E_2 + 96$ h treated mink, uterine glycogen concentrations were higher than controls ($P \leq 0.05$), but approximately 50% less than for $E_2 + 24$ h treated mink ($P \leq 0.05$). For $E_2 \rightarrow P_4$ treated mink, uterine glycogen content was 70% less than for $E_2 + 24$ h treated animals ($P \leq 0.05$) and 40% less than mink treated as $E_2 + 96$ h. Treatment with $P_4 + 24$ h had no effect on gross uterine glycogen content when compared to controls.

Table 2. Mean (\pm SE) uterine endometrial and myometrial areas (pixels $\times 10^4$). Mink in Groups 2, 3 and 4 were each injected once daily on days 12, 13 and 14 with estradiol 17beta (E_2 ; 50 μ g/kg body weight). On the same days, mink in group 5 each received daily injections of progesterone (P_4 ; 25mg/kg body weight). Mink in group 3 received vehicle only on days 15, 16 and 17 while mink in group 4 received daily injections of P_4 on the same days. Mink in group 1 received vehicle only on days 12, 13 and 14 and represented controls. All mink were sacrificed approximately 24 h after the last perturbation.

Group	Treatment	Endometrial area, (pixels $\times 10^4$)	Myometrial area (pixels $\times 10^4$)
1	Control	3.87 \pm 0.65 ^a	7.13 \pm 0.57 ^a
2	$E_2 + 24$ h	16.14 \pm 1.83 ^b	24.96 \pm 4.00 ^b
3	$E_2 + 96$ h	24.87 \pm 3.64 ^c	19.58 \pm 1.20 ^{bc}
4	$E_2 \rightarrow P_4$	11.55 \pm 1.37 ^{ab}	20.31 \pm 4.14 ^{bc}
5	$P_4 + 24$ h	8.59 \pm 0.45 ^{ab}	9.67 \pm 0.54 ^{ac}

^{a-c}Within a column, means without a common superscript differ ($P \leq 0.05$)

Table 3. Mean (\pm SE) glycogen concentrations (mg/g dry weight) in whole uteri, and percent glycogen content of endometrium and myometrium (% area periodic acid-Schiff (PAS) positive \times 10). Mink in groups 2, 3, and 4 were each injected once daily, on days 12, 13 and 14 with estradiol 17beta (E_2 ; 50 μ g/kg body weight). On the same days, mink in group 5 each received daily injections of progesterone (P_4 ; 25 mg/kg body weight). Mink in group 3 received vehicle only on days 15, 16 and 17 while mink in group 4 received daily injections of P_4 on the same days. Mink in group 1 received vehicle only on days 12, 13 and 14 and represented controls. All mink were sacrificed approximately 24h after the last perturbation. To differentiate between mink treated with E_2 only, E_2+24h and E_2+96h refers to uteri collected at 24 and 96 h after the last E_2 injection. Relative fold-increase above controls are given in parentheses.

Group	Treatment	Glycogen (mg/g dry weight)	Endometrial glycogen (% Area PAS+)	Myometrial glycogen (% Area PAS+)
1	Control	2.72 ± 0.29^a	0.62 ± 0.39^a	2.79 ± 1.57^a
2	$E_2 + 24h$	21.91 ± 3.09^b (8)	25.00 ± 4.77^b (40)	25.50 ± 4.82^b (9)
3	$E_2 + 96h$	11.27 ± 2.04^c (4)	17.39 ± 2.75^b (28)	15.70 ± 3.67^b (5)
4	$E_2 \rightarrow P_4$	6.80 ± 0.44^c (2)	4.39 ± 1.33^a (7)	1.69 ± 0.31^a
5	P_4	3.79 ± 0.54^a	0.02 ± 0.01^a	1.68 ± 0.57^a

^{a-c}Within a column, means without a common superscript differ ($P \leq 0.05$).

Table 4 Mean (\pm SE) percent glycogen content of uterine glandular epithelia, luminal epithelia and stroma (% area periodic acid-Schiff (PAS) positive \times 10). Mink in groups 2, 3, and 4 were each injected once daily, on days 12, 13 and 14 with estradiol 17beta (E_2 ; 50 μ g/kg body weight). On the same days, mink in group 5 each received daily injections of progesterone (P_4 ; 25 mg/kg body weight). Mink in group 3 received vehicle only on days 15, 16 and 17 while mink in group 4 received daily injections of P_4 on the same days. Mink in group 1 received vehicle only on days 12, 13 and 14 and represented controls. All mink were sacrificed approximately 24h after the last perturbation. Relative fold-increase above controls are given in parentheses.

Group	Treatment	Glandular epithelia	Luminal epithelia	Stroma
1	Control	0.27 ± 0.15^a	0.54 ± 0.30^a	0.089 ± 0.014^a
2	$E_2 + 24h$	3.63 ± 0.71^b (13)	2.82 ± 1.03^b (5)	0.055 ± 0.013^a
3	$E_2 + 96h$	5.71 ± 0.55^c (20)	11.54 ± 2.32^c (21)	0.141 ± 0.036^b
4	$E_2 \rightarrow P_4$	0.61 ± 0.16^a (2)	0.51 ± 0.13^a	0.054 ± 0.018^a
5	P_4	0.01 ± 0.01^a	0.14 ± 0.05^a	0.013 ± 0.008^c

^{a-c}Within a column, means without a common superscript differ ($P \leq 0.05$)

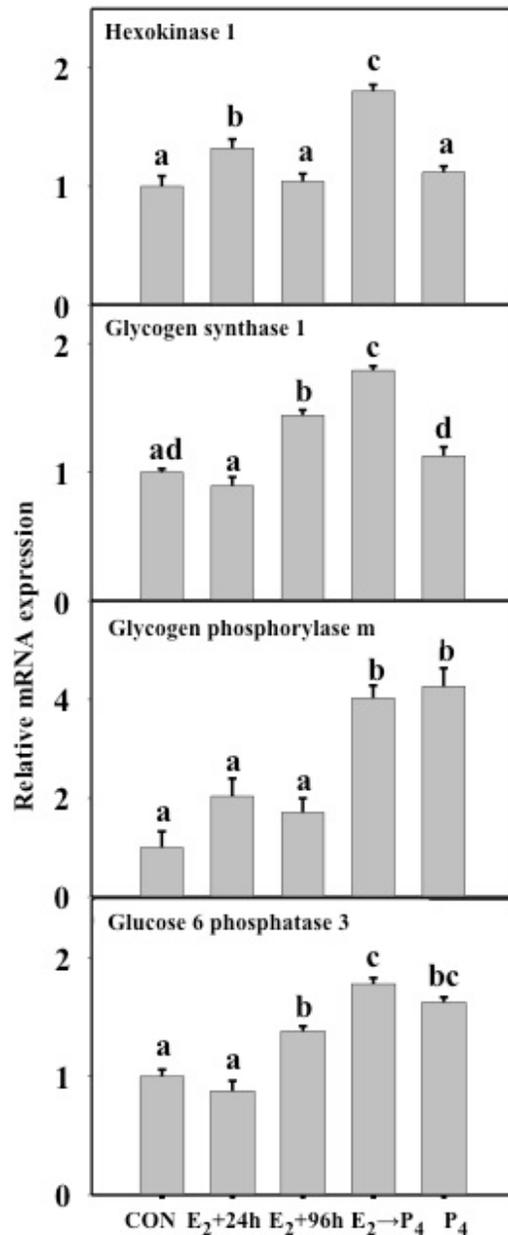


Fig. 3. Relative (mean \pm SE) messenger RNA (mRNA) expression levels for hexokinase 1, glycogen synthase 1, glycogen phosphorylase m and glucose 6-phosphatase in mink uterine homogenates. Animals were injected with E₂ for three days and sacrificed 24 h (E₂ + 24) and 96 h (E₂ + 96) after the last injection. To simulate E₂ priming, mink were treated for three days with E₂ and then an additional three days with P₄ and sacrificed 24 h later (E₂→P₄). Mink were also treated with P₄ for three days and sacrificed 24 h later (P₄ + 24). Controls (CON) received injections of vehicle only for three days and were sacrificed 24 h later. Expression levels represent the mean of five mink, each analyzed in triplicate. Groups without a common letter differ at $P \leq 0.05$.

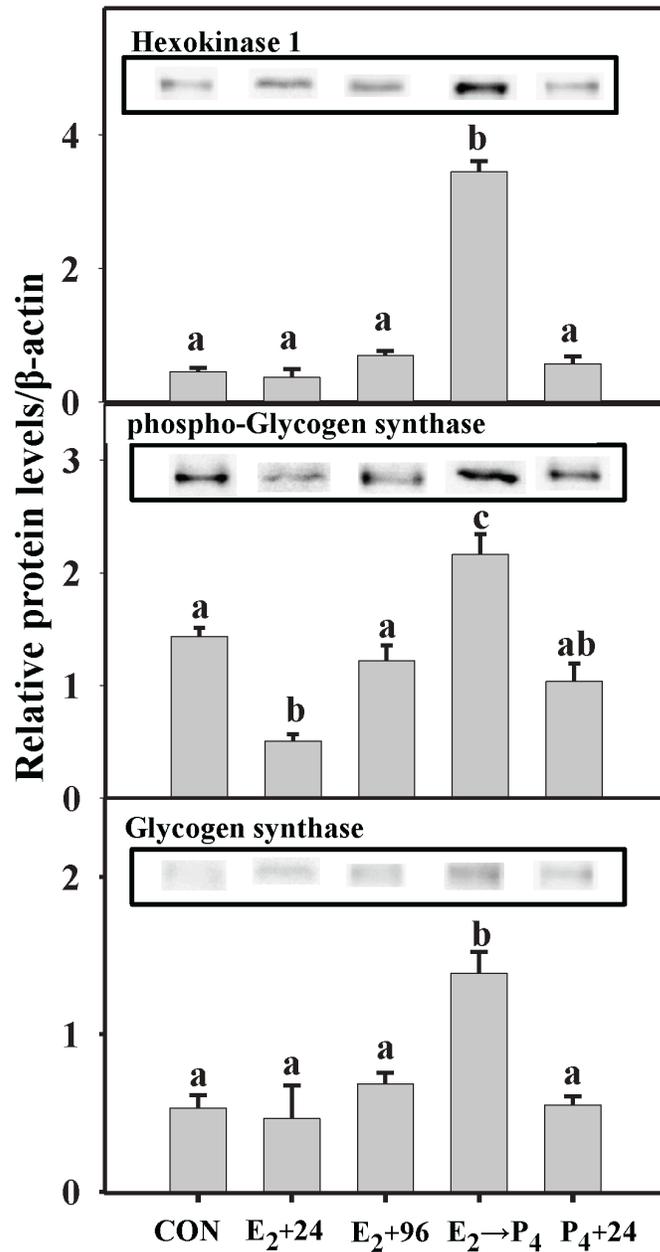


Fig. 4. Western blot analyses for hexokinase 1, phospho-glycogen synthase, and glycogen synthase proteins in mink uterine homogenates. Animals were injected with E₂ for three days and sacrificed 24 h (E₂ + 24) and 96 h (E₂ + 96) after the last injection. To simulate E₂ priming, mink were treated for three days with E₂ and then an additional three days with P₄ and sacrificed 24 h later (E₂→P₄). Mink were also treated with P₄ for three days and sacrificed 24 h later (P₄ + 24). Controls (CON) received injections of vehicle only for three days and were sacrificed 24 h later. Expression levels represent the mean of five mink, each analyzed in triplicate. Groups without a common letter differ at P ≤ 0.05.

ImageJ analyses revealed that both endometrial and myometrial glycogen content were higher in E₂ + 24 h and E₂ + 96 h treated mink, when compared to all other treatments (Table 3; P ≤ 0.05). The glycogen content of both tissues tended to be lower in E₂ + 96 h mink when compared to E₂ + 24 h animals but the differences were not significant. Mink treated as E₂→P₄ had endometrial and myometrial glycogen concentrations that did not differ from controls or mink treated with P₄ + 24 h.

Glycogen content of the glandular and luminal epithelia was highest in E₂ + 96 h mink when compared to all other treatments (Table 4; P ≤ 0.05). In E₂ + 24 h mink, glycogen content of both epithelia was less than for E₂ + 96 h mink, but higher than mink treated with P₄ + 24 h, E₂→P₄ or as controls (P ≤ 0.05). There was no difference in glycogen content of glandular and luminal epithelia among mink treated as E₂→P₄, P₄ + 24 h or controls. Stromal cell glycogen content was highest in E₂ + 96 h treated mink when compared to all other treatments (P ≤ 0.05). There was no difference in stromal cell glycogen content among mink treated as E₂→P₄, E₂ + 24 h or controls. Stromal glycogen content was lowest in response to P₄ + 24 h, when compared to all other groups (P ≤ 0.05).

Uterine *Hkl* mRNA expression in E₂ + 24 h mink was 30% greater than controls (Fig. 2; P ≤ 0.05). There was no difference in expression of the gene in E₂ + 96 h mink or in response to P₄ + 24 h, when compared to controls. When mink were primed with E₂ and subsequently treated with P₄ (E₂→P₄) *Hkl* mRNA expression was higher than for all other treatments (P ≤ 0.05).

Uterine *Gys1* mRNA expression was greatest in response to E₂→P₄ when compared to all other treatments, followed by E₂ + 96 h and P₄ + 24 h (Fig. 3; P ≤ 0.05).

Mink treated as E₂ + 24 h exhibited *Gys1* mRNA expression levels that did not differ from controls.

Expression of *Pygm* mRNA in whole uterine homogenates for E₂ + 24 h and E₂ + 96 h treated mink did not differ from controls (Fig. 3). Mink primed with E₂ and then treated with P₄ (E₂→P₄) or with P₄ + 24 h had higher *Pygm* gene expression levels when compared to mink treated as E₂ + 24 h, E₂ + 96 h or as controls (P ≤ 0.05).

Uterine *G6pc3* mRNA expression was greater for E₂ + 96 h treated mink when compared to E₂ + 24 h or controls (Fig. 3; P ≤ 0.05). Mink treated as E₂→P₄ showed the highest level of *G6pc3* gene expression, followed by treatment with P₄ + 24 h.

The amount of Hk1 and Gys proteins in whole uterine homogenates for mink treated as E₂ + 24 h and E₂ + 96 h or in response to P₄ + 24 h did not differ from controls (Fig. 4). The amount of both proteins in response to E₂→P₄ was greater when compared to all other treatments (P ≤ 0.001). Similarly, the amount of pGys protein was greater in response to E₂→P₄ when compared to all other treatments (P ≤ 0.05). Mink treated as E₂ + 24 h had lower pGys protein content when compared to E₂→P₄ or E₂ + 96 h treatments (P ≤ 0.05), and tended to be lower than mink treated with P₄ + 24 h.

DISCUSSION

Glycogen content of the mink uterine myometrium, endometrium, stroma, luminal and glandular epithelia were increased by exogenous E₂ at 24 and 96 h after the last injection when compared to controls (Tables 3 and 4, Fig. 2; P ≤ 0.05). These findings support our previous observations for mink (Rose *et al.* 2011) and agree with those for rats, rabbits and guinea pigs (Bitman and Cecil, 1967; Bitman *et al.* 1965; Demers *et al.* 1973a; Gregoire *et al.* 1967; Hall and Khaligh 1968; Tripathi 1983).

Because circulating E₂ concentrations in mink are increasing for weeks prior to proestrus and estrus (Pilbeam *et al.* 1979), it would appear that part of the effects of E₂ on the mink uterus are to teleologically, promote glycogen accumulation in preparation for the breeding season. Our recent findings of peak uterine glycogen content in mink during estrus, that diminished throughout implantation and pregnancy (Dean *et al.* 2014) supports this hypothesis. It would not seem unreasonable to propose that without adequate uterine glycogen reserves and their subsequent mobilization, mink pre-embryos may fail to survive and/or develop to the blastocyst stage. This could have a very negative impact on resulting litter sizes.

The glycogen content of whole uteri of E₂ + 96 h treated mink was less than for E₂ + 24 h treated animals (Table 4; P ≤ 0.05). Similarly, the glycogen content of the endometrium and myometrium exhibit the same trend, although the differences were not significant. Such findings might suggest that glycogen catabolism predominated over glycogen synthesis between hours 24 and 96 and that elevated E₂ concentrations above some critical level are required to maintain a higher glycogen synthesis-to-catabolism ratio. And yet, the glycogen content of the glandular epithelia, luminal epithelia and stroma were greater in mink treated as E₂ + 96 h than E₂ + 24 h (Table 4; P ≤ 0.05). We reasoned that glycogen determinations made by ImageJ analysis of the endometrium, myometrium, stroma and whole uterine glycogen measurements were low at 96 h after the last E₂ injection, as a result of being diluted by the relatively large amount of extracellular material and cells not containing glycogen (Fig. 2). As mentioned previously, the endometrial area was approximately four-fold greater at 24 h and six-fold greater at 96 h after the last E₂ injection (Table 2; P ≤ 0.05), when compared to controls.

Even after using ImageJ to isolate the stromal compartment of the endometrium, the glycogen content for the stroma was orders of magnitude below that detected for the glandular and luminal epithelia. Because of the small amount of glycogen detected within such a large area, we believe that our glycogen content measurements for the stroma may have approached the limits of detection by ImageJ analysis and may not be an accurate representation of glycogen content of the stromal cells. By contrast, the epithelia can be very precisely delineated with ImageJ. As a result we believe that glycogen content determinations for glandular and luminal epithelia are the most accurate and physiologically meaningful measurements, since the epithelia is the immediate source of uterine histotroph.

The glycogen content of the glandular epithelia, luminal epithelia and stroma was significantly greater in E₂ + 96 h than E₂ + 24 h treated animals (Table 4). This illustrates that E₂ had prolonged effects on glycogen synthesis in these cells. In agreement with this finding, *Gys1* mRNA levels in uterine homogenates were higher in E₂ + 96 h mink when compared to E₂ + 24 h or controls (Fig. 3; P ≤ 0.05). Although we could detect no difference in total Gys protein by western blot analysis among E₂ + 24 h, E₂ + 96 h and controls (Fig. 4), the amount of pGys protein (inactive) was significantly lower in E₂ + 24 h mink, when compared to controls, which could have contributed to increased glycogen synthesis.

Exogenous E₂ had no effect on *Pygm* gene expression at 24 or 96 h after the last injection (Fig. 3). This agrees with the general consensus that E₂ is glycogenic in the uterus. However, when mink were treated as E₂→P₄ or with P₄ + 24 h, uterine *Pygm* gene expression was higher than E₂ + 24 h, E₂ + 96 h or controls (Fig. 3; P ≤ 0.05).

Moreover, pGys protein and *G6pc3* mRNA were also highest in response to $E_2 \rightarrow P_4$ treatment (Figs 3 and 4). In agreement with this observation we previously showed that expression of *G6pc3* mRNA was five-fold greater during late diapause (as P_4 levels are increasing) and 3.5-fold greater during pregnancy when compared to estrus (Dean *et al.* 2014). Collectively, these findings suggest that P_4 promotes glycogen catabolism and mobilization in the mink uterus.

It is well known that glycogen synthesis and catabolism occur concomitantly within cells and that net glycogen content reflects the relative levels of anabolic and catabolic processes. Thus, even though glycogen reserves in the mink uterus are almost depleted prior to implantation, the level of anabolic activity could still be very high at this time and play a significant role in sequestering maternal glucose to the uterus. In agreement with this supposition, we show that *Hk1* mRNA and Hk1 protein were higher in mink in response to $E_2 \rightarrow P_4$ when compared to all other treatments (Fig 3 and 4). Recently, we showed that Hk1 protein, detected by immunohistochemistry in mink uterine epithelia was high during estrus and peri-implantation (Dean *et al.* 2014). It is possible that increased hexokinase expression in response to P_4 could contribute to increased trapping of glucose from the maternal circulation; even after uterine glycogen reserves are depleted. Similarly, increased Gys expression in response to $E_2 \rightarrow P_4$ (even if catabolism predominated over anabolism), could serve to divert maternal glucose to the uterus. Therefore, it was not surprising to discover that some glycolytic processes in the uterus were enhanced in $E_2 \rightarrow P_4$ treated mink.

In summary, we conclude that mink pre-embryonic survival and development to the blastocyst stage depends in part, upon P_4 induced uterine glycogen catabolism and

release of glucose. During implantation, as circulating E_2 and P_4 concentrations are increasing, P_4 actions may predominate over E_2 , resulting in a greater level of glycogen catabolism than synthesis. Nevertheless, E_2 independently or in combination with P_4 may still promote glycogen synthesis at this time. This latter effect could promote the continued sequestration of glucose from the maternal circulation to the uterus, ensuring adequate nutritional for embryo implantation and early pregnancy. Moreover, we believe that circulating P_4 concentrations must be above some critical level, between mating and implantation, to ensure reproductive success, and maximize litter sizes. As evidence we recently showed that fecal P_4 concentrations from mated female mink increased earlier and were significantly greater in all animals that whelped when compared to those that did not whelp (Cao et al. 2015). We propose that some reproductive failures in mink may be due to (i) an insufficient quantity of glycogen being accumulated by the uterus during estrus; (ii) inadequate mobilization of uterine glycogen reserves after during pre-embryonic development and implantation and/or (iii) failure to divert sufficient amounts of glucose from the maternal circulation to the uterus during early pregnancy. In addition to the many documented actions of P_4 on the uterus, it would now seem that regulation of uterine glycogen metabolism by the steroid should attract the attention of investigators as a potential target for increasing reproductive performance as well as the development of newer and perhaps safer means of birth control.

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Suggested Future Research

1. Some of the most compelling data presented in this thesis is the effects of estrogen and progesterone on the uterine epithelium. These cells are of particular interest as they produce histotrophic nutrition needed by the pre-embryo. However, because whole uterine homogenates were used to detect mRNA and protein expression, these data are unlikely to be representative of the physiology of the epithelial cells. Because immortalized mink uterine epithelial and stromal cells are available, it would be possible to repeat the experimental treatments presented in this thesis, examining the effects of estradiol and progesterone on mRNA and protein expression specifically in the uterine epithelium and stromal cells.
2. Uterine glycogen may be mobilized through catabolism to the individual glucose monomers or exocytosis as whole glycogen molecules, a process hypothesized but not yet observed. The zona pellucida is sufficiently porous to allow the embryo access to large molecules and it is therefore possible that whole glycogen molecules may be endocytosis as an energy source. Future studies would be needed to determine if progesterone initiates glycogen exocytosis from the uterine epithelial cells. Understanding through which pathways glycogen is mobilized would provide insight into possible contraceptive options to regulating glucose secretion.

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