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LUTEOTROPIC EFFECTS OF PROLACTIN ON THE MINK (*Mustela vison*) OVARY DURING EMBRYONIC DIAPAUSE AND EARLY POST-IMPLANTATION GESTATION.

A Thesis Submitted

to the Faculty of Graduate Studies and

Research in Partial Fulfillment of the Requirements

for a Degree of Doctor of Philosophy

in the Department of Animal Science

MacDonald College

McGill University

by

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ABSTRACT

These studies were conducted to determine the mechanisms by which prolactin (PRL) exerts its luteotropic effects on the mink corpus luteum (CL). Three experimental models were developed and utilized in these studies. In the first model, the ovaries from pregnant mink were collected at regular intervals throughout gestation, half the animals were treated with the dopamine agonist 2-bromo-α-ergocryptine (bromocryptine), to suppress their endogenous PRL levels, and half were exposed to their endogenous PRL levels. The second model consisted of treating anestrous animals with exogenous gonadotropins to induce follicular development and ovulation, half the animals were then treated with PRL while the other half were left as untreated controls. In the third model, CL were collected from mink at several stages of mink gestation. The cells were enzymatically dispersed, placed in culture and incubated with different concentrations of PRL, luteinizing hormone (LH), follicle stimulating hormone (FSH) and $(Bu)_2$cAMP. Using these 3 models, the effects of PRL on P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), steroidogenic acute regulatory protein (StAR), luteinizing hormone receptor (LHr) and prolactin receptor (PRLr) mRNA were determined. Messenger RNA levels for P450scc did not vary significantly over the course of mink gestation and treatment of animals with bromocryptine did not alter the abundance. In the anestrous model, treatment of mink with PRL reduced P450scc mRNA levels below that of the untreated control, while treatment of cultured mink luteal cells with increasing concentrations of PRL had no effect on the abundance of P450scc mRNA. The abundance of 3β-HSD mRNA varied over the course of mink gestation. Levels were low during embryonic diapause, increased during CL reactivation and peaked during post-implantation gestation. Treatment of mink with bromocryptine prevented the pre-implantation rise in 3β-HSD mRNA levels. In the anestrous model, animals treated with PRL displayed a transient increase in 3β-HSD mRNA, while treatment of mink luteal cells with PRL caused a dose dependent increase in 3β-HSD mRNA. StAR mRNA levels did not vary in mink ovaries during gestation and treatment with bromocryptine did not alter its abundance. The anestrous model displayed variant levels of StAR mRNA; however, treatment of cultured mink luteal cells with PRL caused a reduction in the abundance of StAR mRNA. The abundance of mRNA for LHr varied during gestation, a transient peak in LHr mRNA was observed at CL reactivation, levels then remained constant. Treatment of animals with bromocryptine reduced LHr mRNA below that of the pretreatment controls. In both the anestrous model and cultured mink luteal cells, treatment with PRL resulted in an increase in the abundance of LHr mRNA. PRLr mRNA levels were low during embryonic diapause but increased during CL reactivation. Changes in the abundance of PRLr mRNA closely paralleled changes in serum PRL levels and receptor binding. Treatment of animals with bromocryptine prevented the pre-implantation rise in PRLr mRNA levels. In the anestrous model, treatment with PRL stimulated a 3-5 fold increase in PRLr mRNA levels relative to the pretreated control level, while treatment of cultured mink luteal cell with PRL had no effect. In conclusion, the mechanism by which PRL exerts its luteotropic effects in the mink is not due to alterations in the abundance of mRNA for the rate limiting steps of progesterone biosynthesis. Prolactin may, however, play a role in altering the direction of steroidogenesis from the Δ^5 to Δ^4 pathway. Important luteotropic effects of PRL include the regulation of LH and PRLr mRNA.

RÉSUMÉ

Ces études ont été menées afin de déterminer le mécanisme par lequel la prolactine (PRL) exerce un effet lutéotrope sur le corps jaune (CJ) du vison. Trois modèles expérimentaux ont été conçus et utilisés dans ces études. Dans le premier modèle, des ovaires de visons gravides ont été récoltés à des intervalles réguliers durant la période de gestation. La moitié des animaux avait été préalablement traitée avec un agoniste de la dopamine, le 2-bromo-α-ergocryptine (bromocriptine) afin de supprimer la prolactine endogène, l'autre moitié, non (témoins). Le second modèle consistait à traiter des animaux en anoestrus avec des gonadotropines exogènes pour induire le développement folliculaire et l'ovulation. Une moitié des animaux a ensuite été traitée avec de la PRL, l'autre, non (témoins). Dans le troisième modèle, les corps jaunes de vison ont été récoltés à différentes étapes de la gestation. Les cellules ont été isolées par une méthode enzymatique, mises en culture puis incubées avec différentes concentrations de PRL, d'hormone lutéinisante (LH), d'hormone folliculostimulante (FSH) et de $(Bu)_2$cAMP. Les effets de la PRL sur l'ARNm de la P450 coupure de la chaîne latérale (P450scc), le 3ß-hydroxystéroïde, la déshydrogénase (3ß-HSD), la protéine de régulation rapide de la stéroïdogénèse (StAR), le récepteur de l'hormone lutéinisante (LHr) et le récepteur de la PRL (PRLr) ont été déterminés à l'aide de ces trois modèles. Les niveaux d'ARN messager de la P450scc n'ont pas varié significativement tout au long de la période de gestation du vison et n'ont pas été modifiés par le traitement à la bromocriptine. Dans le modèle de l'anoestrus, le traitement des visons avec la PRL a amené les niveaux d'ARNm de P450scc en-deçà de niveaux observés chez les témoins tandis que le traitement des cellules lutéales en culture avec des concentrations croissantes de PRL n'a eu aucun effet sur la quantité d'ARNm de P450scc. La quantité d'ARNm de la 3ß-HSD a varié au cours de la gestation du vison. Les niveaux étaient bas durant la période de diapause embryonnaire, augmentaient durant la réactivation du CJ et atteignaient un pic durant la période de postimplantation. Le traitement des visons avec la bromocriptine a empêché l'augmentation des niveaux d'ARNm de 3ß-HSD pendant la période de pré-implantation. Dans le modèle de l'anoestrus, les animaux traités à la PRL ont présenté une augmentation passagère de l'ARNm de 3ß-HSD. Les niveaux d'ARNm de StAR n'ont pas varié dans les ovaires durant la gestation et n'ont pas été modifiés par le traitement avec la bromocriptine. Pour leur part, les animaux du modèle de l'anoestrus ont présenté des niveaux variables d'ARNm de la StAR. Toutefois, le traitement des cellules lutéales de vison en culture avec de la PRL a causé une réduction de l'ARNm de la StAR. La quantité d'ARNm de LHr a varié durant la période de gestation. Un pic éphémère d'ARNm de LHr a été observé lors de la réactivation du CJ, mais les niveaux sont demeurés constants par la suite. Le traitement des animaux avec la bromocriptine a provoqué la diminution de l'ARNm de LHr en-deçà du niveau observé chez les témoins prétraités. Dans les deux modèles (anoestrus, cellules lutéales de vison), un traitement à la prolactine a provoqué une augmentation de l'ARNm de LHr. Les niveaux d'ARNm de PRLr étaient bas durant la diapause embryonnaire, mais ont augmenté durant la réactivation du CJ. La fluctuation des quantités d'ARNm de PRLr était étroitement associée aux différents niveaux de PRL dans le sérum et au taux de liaison au récepteur. Le traitement des animaux à la bromocriptine a empêché l'augmentation des niveaux d'ARNm de PRLr pendant la pré-implantation. Dans le modèle de l'anoestrus, un traitement à la PRL a provoqué une augmentation des niveaux d'ARNm de PRLr par un facteur de 3 à 5 par rapport aux témoins prétraités. Par contre, un traitement des cellules lutéales de vison à la PRL n'a eu aucun effet. En conclusion, le mécanisme par lequel la PRL exerce son effet lutéotrope chez le vison ne résulte pas des fluctuations de l'ARNm des facteurs limitants de la biosynthèse de la progestérone. La prolactine pourrait toutefois jouer un rôle dans le changement de direction de la stéroïdogénèse de la voie Δ^5 à Δ^4. Un effet lutéotrope important de la PRL serait, entre autres, la régulation le l'ARNm de LH et de PRLr.

PREFACE

Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rational and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. Supervisors must attest to the accuracy of such statements at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.

This thesis is dedicated to
William Douglas.

ACKNOWLEDGMENTS

There are many people I need to thank for their help in the completion of this thesis. First of all I wish to sincerely thank the members of my graduate student advisory committee, Drs. B. Murphy, B. Downey, D. Zadworny and R. Farookhi. I am grateful to the "two Bruces" for having had the opportunity to work with them and for their time, patience and input into this project. I would like to thank D. Zadworny for his help with PCR and for the prolactin primers used in these studies. Special thanks to R. Farookhi for his help in the interpritation of this data, the prolactin binding assay and for straightening me out on women and cricket.

I am also grateful to and would like to thank the other members of my laboratory J. Song, M. Dobias Goff and G Moreau. Not only was your help invaluable for the completion of this work but you also made it fun. I would also like to take this opportunity to say a special thank you to Dr. A. Houde, not only for his help with the molecular biology part of this project but also for his friendship and faith.

The completion of this project would not have been possible without the generous gifts from a number of people, including Dr. T. Wise for the porcine P450scc probe, Dr. F Labrie for the human 3β-HSD probe, Dr. D. Stocco for the StAR probe and Dr. D Silverside for LHr PCR primers. I would also like to thank Mr. M. Morrow for the care and feeding of the experimental animals used in this project.

Finally I would like to thank my friends and family. Without your support and love I never would have been able to finish.

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LIST OF ABBREVIATIONS

3β-HSD	3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase
20α-HSD	20α-hydroxysteroid dehydrogenase
aa	amino acid
bp	base pairs
bromocryptine	2-bromo-α-ergocryptine
BSA	bovine serum albumin
cAMP	cyclic adenosine 3',5'-monophosphate
cDNA	complementary deoxyribonucleic acid
CEH	cholesterol ester hydrolase
CL	corpus luteum
cpm	counts per minute
DEPC	diethyl pyrocarbonate
DHEA	dehydroepiandrosterone
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP)
eCG	equine chorionic gonadotropin
EDTA	sodium ethyenediaminetetra acetic acid
FCS	fetal calf serum
FSH	follicle-stimulating hormone
FSHr	follicle-stimulating hormone receptor
GITC	guanidinium isothiocyanate
GnRH	gonadotropin releasing hormone
GH	growth hormone

GHr	growth hormone receptor
hCG	human chorionic gonadotropin
HDL	high density lipoprotein
hypox	hypophysectomized
kb	kilobase(s)
LDL	low density lipoprotein
LH	luteinizing hormone
LHr	luteinizing hormone receptor
mRNA	messenger ribonucleic acid
NSB	non specific binding
nt	nucleotide
P45017α	P450 17α-hydroxylase
P450arom	P450 aromatase
P450scc	P450 side chain cleavage
PBS	phosphate buffered saline
PCR	polymerase chain reaction
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PMA	phorbal 12 myristate-13-acetate
PMSG	pregnant mare serum gonadotropin
PRL	prolactin
PRLr	prolactin receptor
RED	cytochrome P450 reductase
RIA	radioimmunoassay

RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
StAR	steroidogenic acute regulatory protein

1.0 GENERAL INTRODUCTION

The annual reproductive cycle of the mink includes of a long period of anestrous, which lasts from shortly after parturition in May through December. In January, after the winter solstice, the mink enters proestrous, a poorly defined period during which the transition from anestrous to breeding competence is made. The breeding season, defined as the period of time over which the female will allow mating, begins at the end of February and continues for most of the month of March (Hansson, 1947; Enders, 1952; Pilbeam *et al.*, 1979). Individual mink are not in estrus over the entire breeding season; however, once a female enters estrus she will remain so until the end of the breeding season. Copulation, ovulation and pregnancy do not shorten the period of estrus, nor does lack of copulation nor ovulation lengthen it (Enders, 1952; Elofson *et al.*, 1989).

The mink is an induced ovulator i.e. ovulation does not occur in a cyclic fashion, rather it occurs in response to a mating stimulus (Hansson, 1947; Enders, 1952). In induced ovulators, the neuroendocrine events triggered by mating are channeled through the hypothalamic-preoptic-pituitary-ovarian control system which operates under two conditions; 1). a steady state of basal (tonic) operation, which is responsible for follicular development; and 2). a transient phase, which leads to ovulation (Ramirez and Beyer, 1988). Variation exists between species in the pattern of mating behavior necessary to induce an ovulatory surge of LH as well as the timing of the LH peak and ovulation. Although no studies on mating induced secretion of LH have been carried out in the mink, Murphy (1979) stimulated the release of LH from the anterior pituitary of mink using 2 μg of gonadotropin releasing hormone (GnRH). He found that in animals treated early in the breeding season, elevations in LH levels could be detected as soon as 15 minutes after the injection, with peak levels occurring at 45 minutes. Animals treated later in the breeding season displayed a slightly more prolonged response to the GnRH. Estimates of the time interval between mating and ovulation in the mink have been reported to vary from 36-37 hours (Hansson, 1947) to 42-52 hours (Enders, 1952), with variation existing within and among animals (Venge, 1973).

One of the unique characteristics of the mink is that it will mate more than once during the breeding season i.e. it will remate even though it is already pregnant (Hansson, 1947; Enders, 1952). Shackleford (1952) found that in mink, kits can be the result of any of the matings which took place during the breeding season. The most common explanation for the production of varying parentage of litters is a phenomenon known as superfecundation, in which ova released from a single ovulation may be fertilized by any of the matings which took place. The other explanation is superfetation, in which different

matings fertilize ova produced at different ovulations. Both of these phenomenon appear to be common in the mink with the latter being the most predominant (Shackleford, 1952).

Fertilization in the mink takes place in the distal portion of the oviduct and typically occurs 53 hours post coitum (Hansson, 1947; Enders, 1952). The mink embryo divides 6-7 times to form a blastocyst consisting of a hollow ball of 200 to 400 cells. The blastocysts then migrate to the uterus by the 5th or 6th day post coitum (Hansson, 1947). Once the blastocysts reach the uterus they enter a state of embryonic diapause or delayed implantation (Hansson, 1947; Enders, 1952), during which time their development is inhibited and their metabolism restricted to that necessary for the maintenance of life (Mead, 1981). It is this interruption in development that accounts for the great variation in gestation length reported in the mink (Bowness, 1942; Hansson, 1947; Enders, 1952). Development of the embryos resumes when the maternal uterine environment allows implantation. The morphological signs of the completion of diapause are the enlargement of the embryo and its attachment to the endometrium (Sundqvist *et al.*, 1988). The exact mechanism by which dormant embryos become reactivated is not understood, however; it has been correlated to daily changes in photoperiod (Murphy and James, 1974; Travis and Pilbeam, 1980; Murphy, 1984). Experimental lengthening of photoperiod during embryonic diapause has been shown to abbreviate gestation (Pearson and Enders, 1944) through the precocious induction of implantation (Murphy and James, 1974). It is important to note, however, that embryo implantation can still occur in animals devoid of photoreceptors (Murphy and James, 1974) and in animals kept in total darkness (Kirk, 1962).

During embryonic diapause the corpus luteum (CL) of the mink appears histologically inactive (Hansson, 1947) and secretes very low levels of progesterone (Moller, 1973; Murphy, 1973; Stoufflet *et al.*, 1989). It is only after the vernal equinox, as serum prolactin levels are increasing (Pilbeam *et al.*, 1979), that the CL become reactivated and begin to secrete large amounts of progesterone (Murphy *et al.*, 1983). A direct role for prolactin in the reactivation of the mink CL has been suggested since exogenous prolactin will advance the timing of implantation in the intact mink (Papke *et al.*, 1980; Martinet *et al.*, 1981) and prolactin alone has been shown to induce implantation in hypophysectomized mink (Murphy *et al.*, 1981).

Although prolactin has been shown to be essential for the reactivation of the mink CL the exact mechanisms by which it exerts its luteotropic effects are unclear. One hypothesis is that prolactin acts directly on the progesterone biosynthetic pathway. This hypothesis is supported by the fact that prolactin alone can stimulate progesterone production and induce implantation in hypophysectomized mink (Murphy *et al.*, 1981). In addition, prolactin treatment of cultured mink luteal cells, collected from post-implantation mink, has been shown to stimulate small but significant increases in media progesterone

levels (Murphy *et al.*, 1993). Therefore, one of the objectives of this study was to determine the effect of prolactin on several elements of the progesterone biosynthetic pathway, including the steroidogenic enzymes P450scc and 3β-HSD, as well as the cholesterol transport protein, StAR. P450scc along with its electron donors adrenodoxin and adrenodoxin reductase are responsible for the 20-hydroxylation, 22-hydroxylation and cleavage of the C20-C22 bond of cholesterol to produce pregnenolone and isocaproic acid (Reviewed by Miller, 1988; Hinshelwood *et al.*, 1993). Subsequently the oxidation and isomeration of Δ^5-3β-hydroxysteroid pregnenolone into Δ^4-ketosteroid progesterone is carried out by 3β-HSD (Reviewed by Miller, 1988; Hinshelwood *et al.*, 1993). The quantity of progesterone produced by the CL is dependent not only on the activities of these two enzymes, but also upon the provision of cholesterol as substrate. Mobilization of cholesterol from lipid stores to the vicinity of P450scc on the inner mitochondrial membrane is essential for progesterone biosynthesis (Waterman, 1995). The mitochondrial protein StAR, which has recently been purified and cloned by Clark *et al.* (1994), has been reported to be important for the transport of cholesterol across the mitochondrial membrane.

Both LH and prolactin elicit their biological effects by binding to specific cell surface receptors and activating a variety of intracellular signal transduction systems. In addition to determining the effects of prolactin on the progesterone biosynthetic pathway, we also wanted to determine its role in the regulation of luteotropic hormone receptors; Specifically, we wanted to determine the role of prolactin in the regulation of its own receptor as well as the receptor for LH. PRLr is a member of the growth hormone/ prolactin/cytokine receptor family (Kelly *et al.*, 1991). The gene encoding this receptor has been cloned and sequenced in a number of species including the rat (Boutin *et al.*, 1988), mouse (Davis and Linzer, 1989), human (Boutin *et al.*, 1989), rabbit (Edery *et al.*, 1989) and cow (Scott *et al.*, 1992). Multiple forms of the receptor have been identified in mammalian species which differ in the length of their cytoplasmic domain (Davis and Linzer, 1989; Shirota *et al.*, 1990). The deduced amino acid (aa) sequence for the rat prolactin receptor revealed a single polypeptide chain which consists of a large extra-cellular domain of approximately 210 aa, a single transmembrane spanning region of 24 aa and a short (57 aa) or long (262 aa) cytoplasmic domain, depending on the receptor type (Kelly *et al.*, 1991). Recent evidence suggests that there are multiple mechanisms for prolactin signal transduction (Campbell *et al.*, 1994; Clevenger and Medaglia, 1994; Clevenger *et al.*, 1994; Rui *et al.*, 1994; Sidis and Horseman, 1994; Bellanga *et al.*, 1995). LHr is a member of the G protein-coupled hormone receptor family. The gene sequence for LHr has been published for a number of species including the rat (McFarland, 1989), pig (Loosfelt *et al.*, 1989), human (Frazier *et al.*, 1990; Minegishi *et al.*, 1990) and mouse (Gudermann *et al.*, 1992). The deduced aa sequence indicates it is a single polypeptide

chain, a large portion of which is located extra-cellularly, hydropathy plots have revealed a transmembrane domain that weaves through the cell membrane seven times and a short cytoplasmic domain (reviewed by Segaloff and Ascoli, 1993).

In summary, the mink displays a unique combination of reproductive characteristics one of which is an obligatory delay in implantation. During this delay phase the corpus luteum produces little progesterone and it is only after the vernal equinox, as serum prolactin levels are increasing, that the CL become reactivated and begin to produce large amounts of progesterone. The mechanism(s) by which prolactin exerts its luteotropic effects is unknown; therefore, the objectives of this study were 1). to determine the effects of prolactin on the progesterone biosynthetic pathway by monitoring changes in the abundance of mRNA for P450scc, 3β-HSD and StAR, over the course of mink gestation and 2). to determine the effects of prolactin on luteotropic hormone receptors, namely PRLr and LHr. In addition, the possible role of LH in CL reactivation, regulation and maintenance was examined.

2.0 LITERATURE REVIEW

2.1 PROLACTIN AS A LUTEOTROPIN

Astwood (1941) demonstrated the presence of a substance purified from the ovine pituitary gland that maintained the corpus luteum of the hypophysectomized rat. This hormone proved to have neither FSH activity, since it could not induce follicular development in the hypophysectomized rat, nor LH activity, as assessed by its inability to induce ovulation in the rabbit. This hormone became known as prolactin. Prolactin has been shown to regulate a wide spectrum of activities, which include effects on 1). reproduction and lactation, 2). water and salt balance, 3). growth and morphogenesis, 4). metabolism, 5). behavior, 6). immunoregulation and 7). the ectoderm and skin (Nicoll and Bern). In spite of the generality of prolactin's function, the mechanism by which it exerted its actions at a cellular level remained unknown for many years. Recently, however, numerous intra-cellular signal transduction systems have been identified for prolactin (Campbell *et al.*, 1994; Clevenger and Medaglia, 1994; Clevenger *et al.*, 1994; Rui *et al.*, 1994; Sidis and Horseman, 1994; Bellanga *et al.*, 1995).

Prolactin acts as a luteotropin in a wide variety of species, which include examples from every mammalian order. Prolactin is luteotropic in carnivores including the dog (Concannon, 1981), ferret (Murphy, 1979) and mink (Papke *et al.*, 1980; Murphy *et al.*, 1981) as well as some artiodactyls, especially the pig (du Mesnil du Buisson and Denamur, 1969). A luteotropic role for prolactin in ruminant artiodactyls is controversial. The ovine CL regresses following hypophysectomy (Kaltenbach *et al.*, 1968) and the ovine pituitary isolated from the hypothalamus secretes primarily prolactin and little or no LH and can maintain the CL (Denamur *et al.*, 1966). However, treatment of ewes with bromocryptine, which reduces hypophyseal prolactin secretion, has been shown to have no effect on luteal function during the ovine estrous cycle (Niswender, 1974). Treatment of cattle with bromocryptine in combination with passive immunization against prolactin produces no changes in circulating progesterone during the estrous cycle (Hoffmann *et al.*, 1974) leading to the suggestion that prolactin has little or no role in luteal maintenance in the cow.

Evidence for the involvement of prolactin in primate luteal tissues comes from studies of human luteal function in which the characteristic pattern of peripheral progesterone during the menstrual cycle was disrupted by treatment with bromocryptine (Del Pozp *et al.*, 1972; Besser *et al.*, 1972). Administration of bromocryptine to estrogen-treated monkeys resulted in luteolysis, further suggesting that the primate CL requires prolactin (Castracane and Shaikh, 1980).

The luteotropic effects of prolactin has best been studied in the rat. The rat corpus luteum is under the control of both LH and prolactin, with the result that in the absence of either hormone, CL function is lost. In addition, the requirements of the rat CL for LH and

prolactin change over the functional lifespan of the CL (Morishige and Rothchild, 1974). For example, in animals hypophysectomized before day 7, prolactin treatment combined with either estrone (Greenwald and Johnson, 1968), FSH (Greenwald and Johnson, 1968), LH (Yang *et al.*, 1973), or FSH plus LH (Ahmad *et al.*, 1969), has been found to be necessary to maintain pregnancy. LH alone, however, was sufficient to maintain pregnancy in rats hypophysectomized on day 8 or 9 (Ahmad *et al.*, 1969).

The cellular mechanism by which prolactin exerts its effects remains unclear. The principal luteotropic effect of prolactin has been described as a prolongation rather than elevation of the rate of progesterone biosynthesis by the CL (Rothchild, 1981). Prolactin has therefore, been branded as a permissive luteotropin, meaning prolactin permits the CL to secrete progesterone at an intrinsic rate or at a rate determined by other luteotropic factors, such as LH (Rothchild, 1981). Another permissive effect of prolactin is its ability to protect the CL against uterine and other forces that are bent on its dissolution (Ueda *et al.*, 1985; Albarracin and Gibori, 1991). More direct stimulatory effects of prolactin on progesterone biosynthesis such as its role in the regulation of 20α-hydroxysteroid dehydrogenase, cholesterol esterase activity, lipoprotein utilization and the induction and maintenance of LH receptors have also been described and will be reviewed in greater detail below.

2.2 EFFECT OF PROLACTIN ON 20α-HYDROXYSTEROID DEHYDROGENASE

The enzyme 20α-hydroxysteroid dehydrogenase (20α-HSD) plays a key role in the conversion of progesterone to 20α-hydroxyprogesterone, a reduced steroid with weak to nonexistent progestational activity (Talwalker *et al.*, 1966; Rennie and Davis, 1964; Wiest and Forbes, 1964). The CL is a unique steroidogenic gland in that it secretes progesterone, which helps sustain pregnancy, but is also potentially capable of expressing, when required, the enzyme 20α-HSD, which can reduce progesterone to an inactive steroid. Thus, it is crucial for the CL to repress 20α-HSD throughout pregnancy when progesterone is essential for fetal survival and to express this enzyme when pregnancy is to be terminated.

A long known effect of prolactin is to suprress the enzymatic degradation of progesterone to 20α-hydroxyprogesterone (Weist *et al.*, 1968; Jones *et al.*, 1983; Albarracin *et al.*, 1994). Studies in the rat by Albarracin *et al.* (1994) demonstrated that 20α-HSD protein and mRNA levels were coordinately regulated, and that there was a profound inhibitory effect of prolactin on 20α-HSD activity which was apparently due to the inhibition of 20α-HSD gene expression, leading to the disappearance of the protein from the CL. Its imporatant to note, however, that the large scale conversion of progesterone to 20α-hydroxyprogesterone seen in the luteal tissue of the rat (Van Straalan *et al.*, 1981; Kim and Greenwald, 1984) is not universal, since the conversion of large

amounts of progesterone to 20α-hydroxyprogesterone does not appear to occur in the hamster (Harris and Murphy, 1981), mink (Murphy and Moger, 1977) or in a number of other species (Yoshinaga, 1973).

2.3 EFFECT OF PROLACTIN ON CHOLESTEROL ESTER HYDROLASE

Cholesterol esters are present in large concentrations within steroidogenic tissues (Behrman *et al.*, 1971; Hoffman and Fajer, 1973; Trzeciak and Boyd, 1973). Hydolysis of cholesterol ester stores produce cholesterol, which serves as a precursor for steroidogenesis (Armstrong, 1968; Barke *et al.*, 1973; Behrman *et al.*, 1970; Trzeciak and Boyd, 1973), as well as long chain fatty acids, which may provide a major source of energy for cellular metabolism (Flint and Denton, 1970). The enzyme responsible for catalyzing hydrolysis of cholesterol esters into free cholesterol and long chain fatty acids is cholesterol ester hydrolase (CEH).

A number of studies concerning the effects of prolactin on CEH activity have been conducted. Early studies using hypophysectomized, immature, artificially superovulated rats indicated that prolactin increased CEH activity (Behrman *et al.*, 1970b; Behrman *et al.*, 1971). Subsequent studies using mature, pseudopregnant rats confirmed this observation (Klemcke and Brinkley, 1980a; Klemcke and Brinkley, 1980b). In addition, treatment of mature pseudopregnant rats with bromocryptine, to block the endogenous nocturnal prolactin surge, caused significant reductions in both luteal CEH activity as well as progesterone levels (Klemcke and Brinkley, 1980b). Cholesterol ester hydrolase activity in the rat ovary appears to be regulated at both the transcriptional and translational level (Aten *et al.*, 1995). Removal of prolactin from the circulation by either hypophysectomy or bromocryptine treatment is sufficient to significantly reduce CEH mRNA, protein and activity, whereas treatment with prolactin following hypophysectomy and bromocryptine treatment blocked the reduction of CEH mRNA, protein and activity (Aten *et al.*, 1995). Changes in CEH mRNA with luteinization and functional luteal regression are also consistant with a major role for prolactin in the regulation of CEH (Aten *et al.*, 1995).

The mechanism of regulation of CEH activity by prolactin is not known. Prolactin may directly influence CEH transcription rates, although there are no indication that the CEH gene contains a prolactin response element (Small *et al.*, 1991). Alternatively, prolactin may increase CEH mRNA stability or may stimulate protein synthesis by increasing elongation factor 2 activity (Gibori,1993).

2.4 EFFECT OF PROLACTIN ON LIPOPROTEINS

The major source of cholesterol for luteal progesterone synthesis in the rat has been shown to be circulating lipoprotein cholesterol (reviewed by: Strauss *et al.*, 1981 and Gwynne and Strauss, 1982). Exogenous lipoproteins supplied to luteal cells *in vitro* have been shown to augment progesterone secretion in cells derived from the CL of the rat (Schuler *et al.*, 1981), cow (Pate and Condon, 1982), woman (Carr *et al.*, 1981), pig (Grinwich *et al.*, 1983), mink (Murphy *et al.*, 1984) and ferret (McKibbin *et al.*, 1984). There are two forms of lipoproteins which are preferentially utilized in luteal progesterone biosynthesis, high density lipoproteins (HDL) and low density lipoproteins (LDL). Both HDL and LDL can contribute cholesterol to the rat CL (Bruot *et al.*, 1982; Azhar and Menon, 1981); however, HDL appears to be the more significant source of substrate (Schuler *et al.*, 1981). In most other species studied, LDL has been shown to be more effective at supporting progesterone biosynthesis (Gwynne and Strauss, 1982).

Evidence for prolactin's involvement in lipoprotein utilization has been demonstrated by interactions between prolactin and lipoprotien in production of progesterone by luteal cells *in vitro*. Prolactin enhances both HDL- and LDL-induced progesterone accumulation by luteal cells from the pregnant pig (Rajkumar *et al.*, 1984; Chedrese *et al.*, 1988), mink (Murphy *et al.*, 1984) and ferret (McKibbin *et al.*, 1984). Furthermore, an increase or reduction in circulating prolactin in the ferret has been shown to bring about an increase or reduction in the capacity of the subsequent luteal cells to utilize lipoproteins (Rajkumar *et al.*, 1987). The mechanism of this interaction is not clear; however, it has been shown that prolactin increases both the binding and degradation of LDL (reviewed by Murphy and Silivan, 1989).

Murphy *et al.* (1989) tested the effects of LH and prolactin on the uptake of labelled lipoproteins *in vivo* in immature rats treated with gonadotropins to induce ovulation and luteinization. Animals underwent hypophysectomy after the ovulatory stimulus and were treated with 100 µg of LH or prolactin for 5 days. The rats were then infused with labelled HDL and killed after 1 hour. The results revealed that peripheral progesterone was maintained by prolactin but not LH treatment in hypophysectomized rats relative to the sham controls. Both LH and prolactin resulted in HDL uptake by luteal tissue. They concluded that these results suggested that a component of luteal maintenance by luteotropins consisted of support of one or more phases of the cholesterol imporation system of luteal cells.

2.5 EFFECT OF PROLACTIN ON LUTEINIZING HORMONE RECEPTOR

Luteinizing hormone is an important substance produced by the anterior pituitary which is involved in the regulation, maintenance and function of the mammalian CL. This hormone exerts its biological effects by binding to a cell surface receptor, which is a member of the G protein-coupled hormone receptor family. One of the luteotropic functions of prolactin is its involvement in the regulation of LHr.

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has a decisive role in the functional regression of the corpus luteum in most species including the rat (Olofsson and Leung, 1994). Previous studies have shown that one consequence of a luteolytic dose of $PGF_{2\alpha}$ is the disappearance of LH binding capacity from the luteal membrane (rat: Grinwich *et al.*, 1976; sheep: Diekman *et al.*, 1978). Grinwich *et al.* (1976) reported that LHr loss induced by $PGF_{2\alpha}$ could be reversed by pretreatment of rats with prolactin. In addition, Bjurulf *et al.* (1994) found that prolactin could sustain CL function during the late luteal phase by stimulating both progesterone production and the expression of LH receptors. Taken together these data suggest that prolactin is involved in the maintenance of LHr in this species (Grinwich *et al.*, 1976). The inhibition of prolactin secretion during the early luteal phase of the rat has been shown to result in a decrease in LH binding capacity in luteal tissue (Grinwich *et al.*, 1976; Holt *et al.*, 1976), as well as a reduction in the expression of LHr mRNA (Gafvels *et al.*, 1992).

Other modes of action of prolactin in the maintenance of LH receptors may be related to the availability of existing receptors. Luteolysis induced by endogenous means or exogenous $PGF_{2\alpha}$ alters the physical state of the luteal membranes (Buhr *et al.*, 1979). This alteration, a phase change in the phospholipid bilayer of the cell membrane and an increase in membrane viscosity, can be reversed by pretreatment with prolactin (Buhr *et al.*, 1983). In other membrane systems, increases in membrane fluidity are associated with increased prolactin receptor binding (Bhattacharya and Vonderhaar, 1979). Milvae *et al.* (1983) demonstrated that methylation of phospholipids in luteal membranes, a treatment that increases membrane fluidity in turkey erythrocytes (Rimon *et al.*, 1978), increased LH-stimulated progesterone secretion *in vitro*. While other mechanisms of increased target organ sensitivity to peptidergic hormones can be envoked by increased membrane fluidity (Rimon *et al.*, 1978). Milvae *et al.* (1983) proposed that increased membrane fluidity may unmask LH receptors. The prolactin maintenance of LH receptor may then be partly implemented by the regulation of membrane viscosity.

VARIATION IN LUTEOTROPIC HORMONE RECEPTORS IN THE OVARY OF THE MINK (*Mustela vison*) DURING EMBRYONIC DIAPAUSE AND EARLY POST-IMPLANTATION GESTATION.

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Key Words: Mink, embryonic diapause, prolactin, prolactin receptor, LH receptor.

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3.2 ABSTRACT

Prolactin (PRL) is the principal luteotropic hormone in the mink. The role of luteinizing hormone (LH) in maintaining the mink corpus luteum (CL) has not been clearly established. The objectives of this study were to clone cDNA probes for the mink luteotropic hormone receptors and determine changes in the relative abundance of their mRNA over the course of gestation, particularly at the time of CL reactivation and the pre-implantation rise in progesterone. A 675 base pair (bp) fragment from the extra-cellular and transmembrane domain of the mink PRLr gene was amplified, cloned and sequenced. It displayed nucleic acid sequence homology of 78, 79, 81, 84 and 86% with similar regions the rat, murine, human, bovine and rabbit PRLr gene respectively and 53-54% homology with the rat, human and rabbit growth hormone receptor (GHr) gene. A 1056 bp fragment from the transmembrane and cytoplasmic regions of the mink LHr gene was amplified, cloned and sequenced. It displayed nucleic acid sequence homology of 84, 85, 90 and 91% with the corresponding regions of the murine, rat, porcine and human LHr genes respectively and 64-65% homology with the human, porcine and bovine follicle stimulating-hormone receptor (FSHr) gene. To determine changes in the abundance of mRNA for these genes, ovary and serum samples were collected from pregnant female mink every three days beginning on March 19 through to March 31 and then every five days until April 15. One group of animals was implanted with Alzet osmotic minipumps which released 2 mg of the dopamine agonist 2-bromo-α-ergocryptine (bromocryptine) per day. The second group acted as an untreated control. Northern analysis revealed that the PRLr probe bound to three transcripts in the mink which were 3.4, 4.4 and 10.5 kb in size. PRLr mRNA was low in the ovary during the period associated with CL reactivation but increased 3 fold as the time of implantation approached. Circulating prolactin and prolactin binding followed a similar pattern. Treatment of animals with bromocryptine, to suppress endogenous prolactin levels, prevented the increase in PRLr mRNA. The abundance of ovarian LHr mRNA peaked early between March 19 and March 23, declined to basal levels by March 25 and remained constant through mid gestation. Bromocryptine treatment prevented the pre-implantation peak in LHr mRNA and reduced its abundance to below pre-treatment controls. It is concluded that prolactin up-regulates its own receptor and increases LHr in the mink ovary. In addition, changes in the pattern of LHr mRNA suggest that LH may be involved in CL reactivation and the termination of embryonic diapause.

3.3 INTRODUCTION

Gestation in the mink is characterized by an obligate period of embryonic diapause (Hansson, 1947; Enders, 1952). During the delay phase the mink corpus luteum secretes low levels of progesterone (Moller, 1973). It is only after the vernal equinox that the mink CL is reactivated and begins to secrete elevated progesterone (Allais and Martinet, 1978; Pilbeam *et al.*, 1979). Mustelids including the ferret (Murphy, 1979), spotted skunk (Mead, 1975), and the mink (Murphy and Moger, 1977; Murphy *et al.*, 1980) require an intact hypophysis for normal luteal function. In the mink, the pituitary is necessary, not only for luteal reactivation and implantation (Murphy and Moger, 1977), but also for luteal maintenance after implantation (Murphy *et al.*, 1980). A number of studies have determined that the pituitary hormone prolactin is the principal luteotropic hormone in the mink. It is essential for reactivation of the CL, termination of embryonic diapause and for normal CL function after implantation (Papke *et al.*, 1980; Martinet *et al.*, 1981; Murphy *et al.*, 1981). The mechanism(s) by which prolactin exerts its luteotropic effects is unclear. Previous studies have indicated that LH may not be essential for the reactivation of the CL or the termination of embryonic diapause (Murphy *et al.*, 1981), but may be involved in the maintenance of the post-implantation CL (Murphy *et al.*, 1993). To date, its role has not been clearly defined.

Prolactin and LH bind to hormone cell surface receptors and activate specific second messenger systems. Prolactin receptor is a member of the GH/PRL/cytokine receptor family (Kelly *et al.*, 1991). The gene encoding this receptor has been cloned and sequenced in a number of species including the rat (Boutin *et al.*, 1988), mouse (Davis and Linzer, 1989), human (Boutin *et al.*, 1989), rabbit (Edery *et al.*, 1989) and cow (Scott *et al.*, 1992). Multiple forms of the receptor have been identified in mammalian species which differ in the length of their cytoplasmic domain (Davis and Linzer, 1989; Shirota *et al.*, 1990). The deduced amino acid (aa) sequence for the rat prolactin receptor revealed a single polypeptide chain which consists of a large extra-cellular domain of approximately 210 aa, a single transmembrane spanning region of 24 aa and a short (57 aa) or long (262 aa) cytoplasmic domain, depending on the receptor type (Kelly *et al.*, 1991). Recent evidence suggests that there are multiple mechanisms for prolactin signal transduction (Campbell *et al.*, 1994; Clevenger and Medaglia, 1994; Clevenger *et al.*, 1994; Rui *et al.*, 1994; Sidis and Horseman, 1994; Bellanga *et al.*, 1995).

Luteinizing hormone receptor is a member of the G protein-coupled hormone receptor family. The gene sequence for LHr has been published for a number of species including the rat (McFarland, 1989), pig (Loosfelt *et al.*, 1989), human (Frazier *et al.*, 1990; Minegishi *et al.*, 1990) and mouse (Gudermann *et al.*, 1992). The deduced aa

sequence indicates it is a single polypeptide chain, a large portion of which is located extracellularly and hydropathy plots have revealed a transmembrane domain that weaves through the cell membrane seven times and is followed by a short cytoplasmic domain (reviewed by Segaloff and Ascoli, 1993).

Since prolactin and LH are necessary for the maintenance and function of the mink corpus luteum, it was of interest to determine changes in the abundance of PRLr and LHr mRNA during CL reactivation and early post-implantation gestation and to determine whether alterations in endogenous prolactin levels affect the abundance of mRNA for either PRLr or LHr.

3.4 MATERIALS AND METHODS

4.4.1 TOTAL RNA ISOLATION

Ovarian and testicular total RNA was extracted by CsCl (Gibco/BRL, Burlington, ON) gradient ultracentrifugation (Chirgwin *et al.*, 1979). Tissue samples were homogenized with a PT 3000 polytron (Brinkmann, Rexdale, ON) in 4 M guanidinium isothiocyanate (GITC: Gibco/BRL) plus 0.12 M 2 mercaptoethanol (Sigma, St Louis, MO). The homogenate was then layered onto a 5.7 M CsCl gradient and centrifuged at 32,000 rpm (174,000g) using a SW-41 rotor (Beckman, Mississauga, ON), for 20 hours at 22 C. The RNA pellet was then dissolved and precipitated twice in 0.1 volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol. After the final washing the pellet was dissolved in diethyl pyrocarbonate (DEPC: Sigma)-treated distilled water and stored at -70 C. The nucleic acid concentration for each sample was determined by spectrophotometric measurement at 260 nm.

4.4.2 CLONING AND SEQUENCING OF PROBES FOR PRLr AND LHr

Oligonucleotide primers for the reverse transcription (RT) and polymerase chain reaction (PCR) amplifications for the mink PRLr gene were based on homologous regions of the rat (Boutin *et al.*, 1988; Shirota *et al.*, 1990), mouse (Davis and Linzer, 1989), rabbit (Edery *et al.*, 1989) and bovine (Scott *et al.*, 1992) PRLr genes (sense primer **A**: AGGAAACATTCACCTGCTGGTG; antisense primer **1**: TGCATCCTCCCACCAGT TCC). Primers for the mink LHr gene were based on homologous regions of the rat (McFarland *et al.*, 1989), porcine (Loosfelt *et al.*, 1989) and human (Minegishi *et al.*, 1990) LHr genes (sense primer **C:** GGAGAATTCATTTGCCTCCCCATGGATG TGGAA, **C:** GGGAATTCCCTGTGAAGATATTATGGGCTATG; antisense primer **1:** GGTGTCTAGATGCAGAAGCTTGCAAAGGAGAGATT, **dT**: GACTCGAGTCGA CATCGA).

Total ovarian or testicular RNA was reverse transcribed using the following reaction conditions. Five μg of RNA, 1 μl of 25 μM antisense primer and enough DEPC-treated water to bring the total volume to 16 μl were combined. This mixture was incubated at 65 C for 5 min and then put on ice. To the primer:RNA mix 2 μl of 10 x RT buffer (0.5 M Tris, 0.7 M KCl, 0.1 M $MgCl_2$, 0.04 M DTT), 0.5 μl RNAsin (Pharmacia), 1 μl nucleotide mix (25 μM each of dATP, dCTP, dGTP, dTTP), and 0.5 μl murine Moloney leukemia virus reverse transcriptase (Pharmacia) were added for a total volume of 20 μl. The reaction mixture was incubated for 30 minutes at 42 C, 30 minutes at 45 C and 30 minutes at 47 C. Polymerase chain reaction amplifications were performed using sense and antisense primers for each gene. Reactions were made to a total volume of 100 μl and contained 10 μl of 10 x PCR buffer (0.5 M Tris pH 9, 15 mM $MgCl_2$, 0.2 M NH_2SO_4), 1 μl of each of the sense and antisense primers, 1 μl of 20 μM of each nucleotide (dNTP: Pharmacia), 0.5 μl of cDNA pool and 0.5 μl TAQ polymerase (Pharmacia). The PCR reactions were performed in a thermocycler for 40 cycles.

The PCR products were size fractionated by electrophoresis on 1% agarose gels. The gels were stained with ethidium bromide (Sigma) and the presence of cDNA bands was determined by visualization under UV illumination. Amplified cDNA bands of the correct predicted size were excised from the agarose gels and purified using sephaglas bandprep (Pharmacia). The purified bands were ligated into pGEM-T (Promega, Nepean, ON) plasmid vector using T4 DNA ligase (Promega). The ligation products were used to transform *E. coli* (J105), using the RbCl method (Sambrook *et al.*, 1989). Positive clones were identified by selection for ampicillin resistance and restriction enzyme digestion of miniprep plasmid DNA preparations. Plasmids containing the proper inserts were sequenced by the double stranded dideoxy chain termination method (Sambrook *et al.*, 1989) using T7 polymerase (T7 Sequencing Kit: Pharmacia). To guard against potential misincorporation of nucleotides by TAQ polymerase during PCR amplification, three independent clones were sequenced for each gene fragment and the consensus sequence taken.

3.4.3 EXPERIMENTAL DESIGN

Adult female mink were maintained on a commercial mink ranch (Morrow Fourrures; St Paul d'Abbotsford, PQ). They received a standard wet mink ration and water *ad libitum*. Beginning on March 3, female mink were exposed to males every two days until mating. Seven to nine days later the females were remated to different males. Successful matings were confirmed by the presence of motile sperm in vaginal smears. The pregnant females were randomly assigned to one of two treatment groups. The first group were implanted on March 20 with Alzet osmotic minipumps (Alza Corp, Palo Alto, CA)

which released 2 mg of bromocryptine (Sigma) per day over the experimental period. The second group received pumps which contained saline and acted as controls. The ovaries and a terminal blood sample were collected every 3 days between March 19 and 31 and every 5 days thereafter until April 15. One ovary from each animal was placed in GITC buffer for total RNA isolation as described above. The other ovary was snap frozen and stored at -70 C to determine receptor binding. In addition, to the ovaries various tissue samples including the uterus, brain, adrenal, kidney and liver were also collected for total RNA isolation.

3.4.4 NORTHERN ANALYSIS

For Northern blot analysis, 15 μg of total RNA was size fractionated on a 1% agarose denaturing formaldehyde gel and stained with ethidium bromide (Sigma) as described by Sambrook *et al.*, 1989. The RNA was transferred by capillary action overnight using 20 x SSC (1 x SSC = 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) to a nylon membrane (Hybond-N: Amersham, Oakville, ON) and then cross-linked to the membrane using GS gene linker UV chamber (BioRad, Mississauga, ON).

Prior to hybridization the membranes were rinsed in 5 x SSPE (1 x SSPE = 180 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) then prehybridized for 30 minutes at 65 C in hybridizing solution (5 x SSPE, 5 x Denhardt's solution: 0.5 % sodium dodecyl sulfate (SDS), 10% dextran sulfate). Denatured herring sperm DNA (Sigma) at a concentration of 10 mg/ml was added to the hybridizing solution and the prehybridization period was continued for at least another three hours. The homologous mink PRLr and LHr probes were labeled by random primer extension (Boehringer Mannheim, Laval, PQ) with [^{32}P]-dCTP (Dupont, Mississauga, ON). Labeled probes were denatured and added to the hybridizing solution. The incubation was continued overnight at 65 C. The following day the membranes were washed twice, 15 minutes per wash, with 2 x SSPE plus 0.1% SDS at room temperature and twice at 65 C. The membranes were sealed in hybridizing bags with a small amount of 5 x SSPE and exposed to Kodak XAR-5 film (Rochester, NY) in cassettes with intensifying screens. All membranes were rehybridized with a human ribosomal 28S probe (Gonzales *et al.*, 1985). Autoradiographic images were digitized and analyzed using Collage software. Results are expressed as a ratio between the cDNA probe of interest and ribosomal 28S. These arbitrary units were then expressed as a percentage of a pooled sample derived from ovaries of female mink collected during anestrous. This sample was present on all of the Northern blots and served as a control.

3.4.5 PROLACTIN RECEPTOR BINDING ASSAY

Ovine prolactin was radiolabeled with ^{125}I (Amersham, Oakville, ON) by the lactoperoxidase method (Thorell and Johansen, 1973). Ovarian samples were thawed and kept on ice. Each sample was homogenized using a small glass tissue homogenizer in 250 µl of phosphate buffered saline (PBS). The homogenizer was rinsed twice with 250 µl of PBS and pooled with the tissue sample. The samples were centrifuged at 3000 rpm at 4C for 20 minutes. The supernatant was removed and discarded. The pellet was resuspended in 500µl of ice cold PBS. All binding determinations were performed in polypropylene tubes which were coated with 10% bovine serum albumin (BSA: Sigma). The assay was carried out in duplicate and each sample had its own nonspecific binding (NSB) tube. Sample (150 µl), assay buffer and labeled prolactin (100,000 cpm) were incubated overnight at room temperature. The next day the samples were centrifuged and the pellet was washed twice with PBS plus BSA and counted on a spectrometer. Protein concentrations were determined (Lowry *et al.*, 1951) and used to standardize the results.

3.4.6 HORMONE ASSAYS

Mink serum progesterone concentrations were determined by liquid-phase radioimmunoassay (RIA) after extraction in 10 volumes of hexane (BDH, Darnstadt). Extraction recoveries ranged between 95-98%. An antiserum raised against 4-pregnen-11α-o-13,20dione hemisuccinate bovine serum albumin, kindly provided by Dr. A Goff (Lafrance and Goff, 1985), was used as the first antibody. Progesterone-11α-glucuronide-[^{125}I] iodotryramine (Amersham, Oakville, ON) was used as radioactive trace and goat anti-rabbit IgG (Prince Laboratories, Toronto, ON) as the precipitating second antibody. The sensitivity of the assay was 6 pg. The intra- and inter-assay coefficients of variation were 12.2 and 12.3%, respectively.

Mink serum prolactin concentrations were determined by a double antibody radioimmunoassay, previously validated for the mink (Murphy *et al.*, 1990). The sensitivity of the assay was 0.3 ng/ml .

3.4.7 STATISTICAL ANALYSIS

Mean + SEM values were calculated for each of the parameters measured. Two way analysis of variance was used to determine differences in serum prolactin and progesterone levels, the abundance PRLr and LHr mRNA over the experimental period and between the bromocryptine and control groups. One way analysis of variance was used to determine differences in prolactin receptor binding over pre-implantation and early

post-implantation gestation. In the presence of significant F values, individual differences between means were determined by the LSD method. A value of $P< 0.05$ was considered significant.

4.5 RESULTS

3.5.1 CLONING OF THE MINK PRLr AND LHr cDNA

A 675 bp fragment of the mink PRLr gene, corresponding to nucleotides 120 to 895 of the rat, human, murine, rabbit and bovine PRLr gene, was cloned and sequenced (Figure 1). The fragment included most of the putative extra-cellular and all of the transmembrane domains. It contained five cysteine residues that are conserved between all the species sequenced to date. The fragment also included two potential N-linked glycosylation sites. The mink PRLr gene had sequence homologies of 78, 79, 81, 84 and 86% with the rat (Boutin *et al.*, 1988, Shirota *et al.*, 1990), murine (Davis and Linzer, 1989), human (Boutin *et al.*, 1989), bovine (Scott *et al.*, 1992) and rabbit (Edery *et al.*, 1989) PRLr gene sequences (Table 1). It had 53-54% homology with the rat (Mathews *et al.*, 1989), human (Leung *et al.*, 1987) and rabbit (Leung *et al.*, 1987) GHr gene. Northern analysis revealed that the cloned mink PRLr probe hybridized with three principal transcripts, which were 3.4, 4.4 and 10.5 kb in size (Figure 3a). A tissue distribution blot showed that the mink PRLr probe hybridized strongly with transcripts in the mink ovary, CL and testes (Figure 2) and weakly with the uterus, adrenal, kidney and liver (data not shown).

A 1056 bp fragment of the mink LHr gene, corresponding to nucleotides 1065 to 2115 of the rat, porcine and human LHr genes, was cloned and sequenced (Figure 2). The fragment included all seven putative transmembrane spanning domains and all of the cytoplasmic portion of the gene. This portion of the mink LHr gene had sequence homology of 84, 85, 90 and 91% with the murine (Gudermann *et al.*, 1992), rat (McFarland *et al.*, 1989), porcine (Loosfelt *et al.*, 1989) and human (Frazier *et al.*, 1990; Minegishi *et al.*, 1990) LHr gene sequences respectively (Table 1) and 64-65% with the rat (Sprengel *et al.*, 1990), human (Minegishi *et al.*, 1991), ovine (Yarney *et al.*, 1993) and bovine (Houde *et al.*, 1994) FSHr gene. Northern blot analysis revealed that the mink LHr probe hybridized with several transcripts, the principle one being 2.4 kb in size (Figure 3b). Hybridization was restricted to ovarian and testicular tissue (Figure 3b).

3.5.2 ABUNDANCE OF PRLr and LHr DURING MINK GESTATION

Serum prolactin levels in the pregnant mink increased gradually through gestation from a low of 14.15 + 3.9 ng/ml on March 19 during diapause to 54.08 + 7.6 ng/ml on April 15 during early post-implantation gestation (Figure 4a). Treatment of pregnant animals with 2 mg of bromocryptine per day, reduced prolactin levels below the pre-treatment controls (13.0 + 7.7 ng/ml on March 19 to 2.8 + 0.7 ng/ml on March 23). Some animals, however, were able to breakout of the bromocryptine suppression of prolactin after March 31. This is reflected in a small but significant increase in serum prolactin levels at this time (4.9 + 1.7 ng/ml on March 29 to 14.4 + 5.0 ng/ml on April 5).

Serum progesterone levels increased gradually in the untreated pregnant mink between March 19 (5.1 + 2.4 ng/ml) and April 15 (78.0 + 7.1 ng/ml) (Figure 4b) indicating the reactivation of the mink CL. Treatment of pregnant animals with bromocryptine prevented this reactivation. Animals that escaped from bromocryptine suppression displayed increases in serum progesterone levels indicating CL reactivation (3.3 + 3.3 ng/ml on March 29 to 35.5 + 18.0 ng/ml on April 10). Animals with increased levels of prolactin and progesterone also had implanted embryos present on April 15.

The abundance of mink PRLr did not significantly differ ($P<0.05$) between March 19 and March 29, which corresponds to the period of CL reactivation in the mink. The PRLr mRNA levels increased 3 fold between March 29 and April 15 (Figure 5a). Prolactin binding sites followed a similar pattern of expression as PRLr mRNA (Figure 6), with no difference between March 19 and 29 then increasing between March 29 and April 15. Suppression of endogenous prolactin levels with bromocryptine prevented the increase in the abundance of PRLr mRNA present in the untreated animals on March 29.

The abundance of mink LHr mRNA showed a different pattern of expression from PRLr. There was a rapid increase in the abundance of LHr mRNA between March 19 and March 23 (Figure 7a). The levels then declined until March 25 and remained constant over the rest of the experimental period. Bromocryptine treatment not only prevented the transient increase in LHr mRNA present in the untreated controls, but also reduced the abundance of LHr mRNA below the pre-treated controls. The animals that escaped from bromocryptine suppression had increased levels of LHr mRNA (Figure 7).

Figure 1. The nucleic acid and predicted amino acid sequence of a 675bp fragment of the mink PRLr gene. The fragment corresponds to nucleotides 120-895 of the rat, mouse, human, bovine and rabbit PRLr gene. Them transmembrane domain is enclosed in the box. Conserved cysteine residues are marked with asterisks and potential N-linked glycosylation sites are underlined.

```
          9          18          27          36          45          54
5' AAG GAA ACA TTC ACC TGC TGG TGG AAA CCT GGG GAA GAT GGA GGG CTT CCC ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Lys Glu Thr Phe Thr Cys Trp Trp Lys Pro Gly Glu Asp Gly Gly Leu Pro Thr

         63          72          81          90          99         108
AAA TAC ACG CTG ACG TAT CAC AAG GAA GGA GAA ACA ACC ACA CAT GAA TGT CCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Lys Tyr Thr Leu Thr Tyr His Lys Glu Gly Glu Thr Thr Thr His Glu Cys Pro

        117         126         135         144         153         162
GAC TAC ATA ACC AGT GGC CCC AAT TCC TGT TAC TTC AAC AAG AAG CAC ACC TCC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Asp Tyr Ile Thr Ser Gly Pro Asn Ser Cys Tyr Phe Asn Lys Lys His Thr Ser

        171         180         189         198         207         216
ATA TGG ACG ATG TAC ATC ATC ACA ATA AAT GCC ACA AAC GAG ATG GGA AGC AGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ile Trp Thr Met Tyr Ile Ile Thr Ile Asn Ala Thr Asn Glu Met Gly Ser Ser

        225         234         243         252         261         270
TCC TCG GAT CCA CGT TAT GTG ACG TTG ACT TAC ATT GTT GAA CCA GAC CCT CCT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ser Ser Asp Pro Arg Tyr Val Thr Leu Thr Tyr Ile Val Glu Pro Asp Pro Pro

        279         288         297         306         315         324
GTG AAC CTA AGT TTG GAA TTA AAA CAG CCA GAA GAC AAA AAA ACA TAC CTG TGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Val Asn Leu Ser Leu Glu Leu Lys Gln Pro Glu Asp Lys Lys Thr Tyr Leu Trp

        333         342         351         360         369         378
ATT AAA TGG TAC CCA CCC ACC CTG GTT GAT GTT AGA TCT GGT TGG CTC ACA CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ile Lys Trp Tyr Pro Pro Thr Leu Val Asp Val Arg Ser Gly Trp Leu Thr Leu

        387         396         405         414         423         432
CAG TAT GAA ATT CGG TTA AAA CCC GAG AAA GCT ACT GAG TGG GAG ACT CAT TTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gln Tyr Glu Ile Arg Leu Lys Pro Glu Lys Ala Thr Glu Trp Glu Thr His Phe

        441         450         459         468         477         486
GCT GGA CTG CAG ACT CAG TTT AAG ATT CTC AGC TTA TAC CCA GGA CAG AAA TAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ala Gly Leu Gln Thr Gln Phe Lys Ile Leu Ser Leu Tyr Pro Gly Gln Lys Tyr

        495         504         513         522         531         540
CTT GTC CAG GTT CGC TGC AAG CCA GAC CAT GGA TTC TGG AGT GAG TGG AGC CCA
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Leu Val Gln Val Arg Cys Lys Pro Asp His Gly Phe Trp Ser Glu Trp Ser Pro

        549         558         567         576         585         594
AAG AGG TCT ATC CAG ATA CCT AAT GAC ATC TCC ATG AAA GAT ACC ATT GTG TGG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Lys Arg Ser Ile Gln Ile Pro Asn Asp Ile Ser Met Lys Asp Thr Ile Val Trp

        603         612         621         630         639         648
ATC TTT GTG GCC GTT CTC TCC GCA GTC ATC TGT CTG ATT ATG GTC GCA GCC GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ile Phe Val Ala Val Leu Ser Ala Val Ile Cys Leu Ile Met Val Ala Ala Val
```

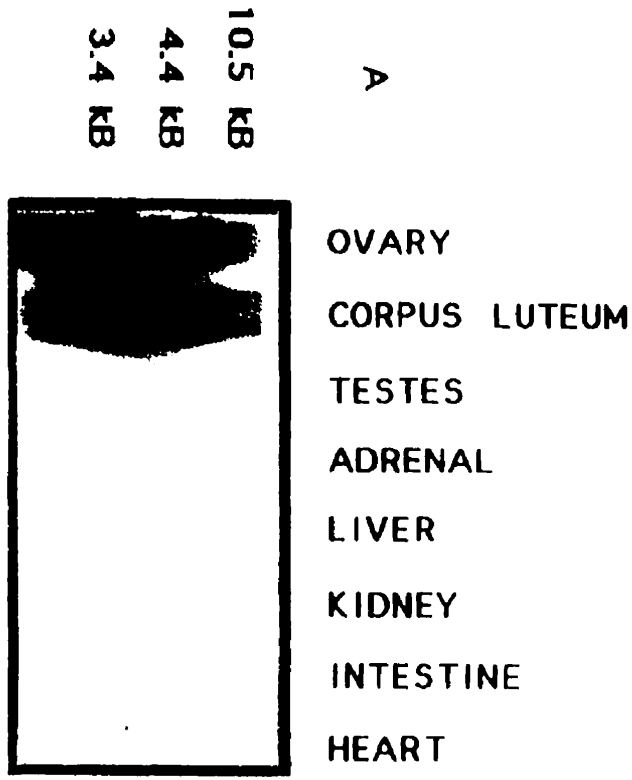
```
         657         666         675
GCT CTG AAA GGC CAT AGC ATG GTC ACC 3'
--- --- --- --- --- --- --- --- ---
Ala Leu Lys Gly His Ser Met Val Thr
```

Figure 2. The nucleic acid and predicted amino acid sequence of a fragment of the mink LHr gene. The fragment corresponds to nucleotides 1065-2115 of the rat, porcine and human LHr gene. The seven transmembrane domains are numbered and enclosed in boxes. Conserved cysteine residues are marked with asterisks and potential phosphorylation sites by dark dots.

```
          10          19          28          37          46          55
5' TTC CTT AGG GTC TTG ATT TGG CTG ATT AAT ATC TTG GCC ATC ATG GGA AAT GTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Phe Leu Arg Val Leu Ile Trp Leu Ile Asn Ile Leu Ala Ile Met Gly Asn Val

          64          73          82          91         100         109
ACT GTT CTC TTT GTT CTC CTG ACT AGT CGC TAT AAA ATG ACG GTG CCC CGT TTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Thr Val Leu Phe Val Leu Leu Thr Ser Arg Tyr Lys Met Thr Val Pro Arg Phe

         118         127         136         145         154         163
CTC ATG TGC AAT CTA TCT TTT GCA GAT TTT TGC ATG GGG TTC TAT CTG CTG CTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Leu Met Cys Asn Leu Ser Phe Ala Asp Phe Cys Met Gly Phe Tyr Leu Leu Leu

         172         181         190         199         208         217
ATT GCC TCA GTT GAT TCC CAA ACC AAA GGC CAA TAT TAT AAC CAT GCC ATA GAC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ile Ala Ser Val Asp Ser Gln Thr Lys Gly Gln Tyr Tyr Asn His Ala Ile Asp

         226         235         244         253         262         271
TGG CAG ACC GGG AGT GGG TGT AGT GCA GCT GGC TTT TTC ACT GTA TTT TCA AGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Trp Gln Thr Gly Ser Gly Cys Ser Ala Ala Gly Phe Phe Thr Val Phe Ser Ser

         280         289         298         307         316         325
GAG CTT TCT GTC TAC ACC CTC ACA GTT ATC ACA CTA GAA AGA TGG CAC ACC ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Glu Leu Ser Val Tyr Thr Leu Thr Val Ile Thr Leu Glu Arg Trp His Thr Ile

         334         343         352         361         370         379
ACC TAT GCT CTT CAG CTG GAC CAA AAG CTA CGT TTA AGA CAT GCC ATA CTG ATC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Thr Tyr Ala Leu Gln Leu Asp Gln Lys Leu Arg Leu Arg His Ala Ile Leu Ile

         388         397         406         415         424         433
ATG CTT GGA GGA TGG CTC TTT TCT ACT CTA ATT GCC ACA TTG CCC CTT GTG GGT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Met Leu Gly Gly Trp Leu Phe Ser Thr Leu Ile Ala Thr Leu Pro Leu Val Gly

         442         451         460         469         478         487
GTC AGC AAT TAC ATG AAG GTC AGC ATT TGC CTC CCC ATG GAT GTG GAA ACC ACT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Val Ser Asn Tyr Met Lys Val Ser Ile Cys Leu Pro Met Asp Val Glu Thr Thr

         496         505         514         523         532         541
CTC TCC CAA GTC TAC ATA TTA ACC ATT CTG ATA CTC AAT GTG GTG GCC TTC ACC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Leu Ser Gln Val Tyr Ile Leu Thr Ile Leu Ile Leu Asn Val Val Ala Phe Thr

         550         559         568         577         586         595
ATC ATC TGT GCT TGC TAC ATT AAA ATT TAT TTT GCA GTT CAA AAT CCA GAG CTG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Ile Ile Cys Ala Cys Tyr Ile Lys Ile Tyr Phe Ala Val Gln Asn Pro Glu Leu

         604         613         622         631         640         649
ATG GCT AAC AAC AAA GAT ACG AAG ATT GCC AAG AAA ATG GCA GTC CTC ATC TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Met Ala Asn Asn Lys Asp Thr Lys Ile Ala Lys Lys Met Ala Val Leu Ile Phe
```

```
        658         667         676         685         694         703
ACT GAT TTC ACT TGC ATG GCA CCA ATC TCC TTT TTT GCC ATC TCA GCT GCC TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Thr Asp Phe Thr Cys Met Ala Pro Ile Ser Phe Phe Ala Ile Ser Ala Ala Phe

        712         721         730         739         748         757
AAA GTG CCC CTT ATC ACA GTA ACC AAC TCT AAA GTT TTA CTG GTT CTT TTT TAT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Lys Val Pro Leu Ile Thr Val Thr Asn Ser Lys Val Leu Leu Val Leu Phe Tyr

        766         775         784         793         802         811
CCT GTC AAT TCT TGT GCC AAT CCA TTT CTG TAT GCG ATT TTC ACA AAG GCA TTC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Pro Val Asn Ser Cys Ala Asn Pro Phe Leu Tyr Ala Ile Phe Thr Lys Ala Phe

        820         829         838         847         856         865
CAA AGG GAT TTC TTT CTG TTG CTG AGC AAA TTT GGC TGC TGT AAA CGT CGG GTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Gln Arg Asp Phe Phe Leu Leu Leu Ser Lys Phe Gly Cys Cys Lys Arg Arg Val

        874         883         892         901         910         919
GAA CTA TAT AGA AGG AAG GAT TTT GTG GCT TAT ACC TCC AAC TGC AAA AAT GGC
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Glu Leu Tyr Arg Arg Lys Asp Phe Val Ala Tyr Thr Ser Asn Cys Lys Asn Gly

        928         937         946         955         964         973
TTC ACT GGA TCA AAT AAG CCT TCT CAG TCC ACC CTG AAG TTG TCT CCA TTG CAG
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Phe Thr Gly Ser Asn Lys Pro Ser Gln Ser Thr Leu Lys Leu Ser Pro Leu Gln

        982         991        1000        1009        1018        1027
TGT CAA TAT ACA ACT GTC CCA GAC AAG ACT TGC TAA TAA AGA GTG TTA ACT GTT
--- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
Cys Gln Tyr Thr Thr Val Pro Asp Lys Thr Cys *** *** Arg Val Leu Thr Val

       1036        1045        1054
TTA TCA GTA ACC ACA AAA AAA AAA AAA AA 3'
--- --- --- --- --- --- --- --- --- --
Leu Ser Val Thr Thr Lys Lys Lys Lys
```

Figure 3. (a). Northern blot showing the tissue distribution of mink PRLr mRNA. The homologous mink PRLr probe hybridized with 3 transcripts 3.4, 4.4 and 10.5 kb in size. (b). Northern blot showing the tissue distribution of mink LHr mRNA. The homologous LHr probe hybridizes to several transcripts, the principal one being 2.4 kb in size.


10.5 kB
4.4 kB
3.4 kB
A
OVARY
CORPUS LUTEUM
TESTES
ADRENAL
LIVER
KIDNEY
INTESTINE
HEART

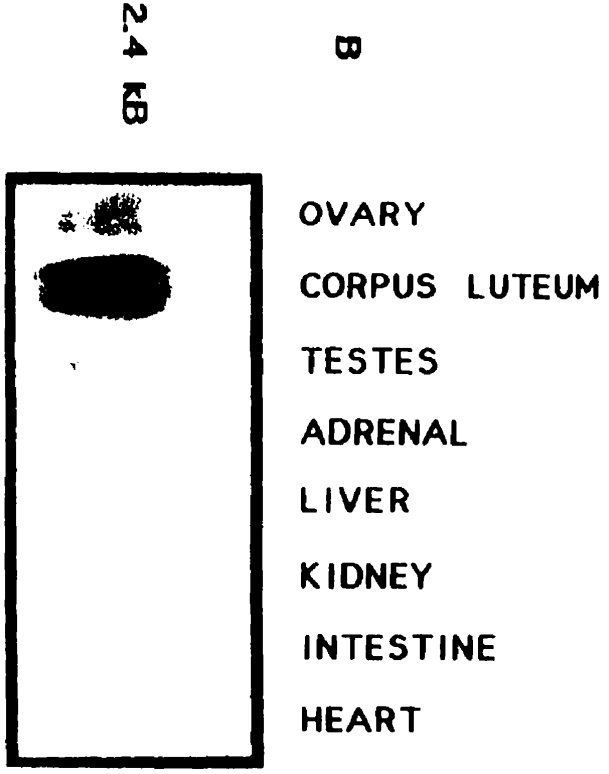

B
2.4 kB
OVARY
CORPUS LUTEUM
TESTES
ADRENAL
LIVER
KIDNEY
INTESTINE
HEART

Figure 4. (a). Mean (+ SEM) of mink serum prolactin levels from embryonic diapause to early post-implantation gestation. Serum prolactin levels were determined by double antibody RIA. (b). Mean (+ SEM) of mink progesterone levels from embryonic diapause to early post-implantation gestation. Serum progesterone levels were determined by double antibody RIA.

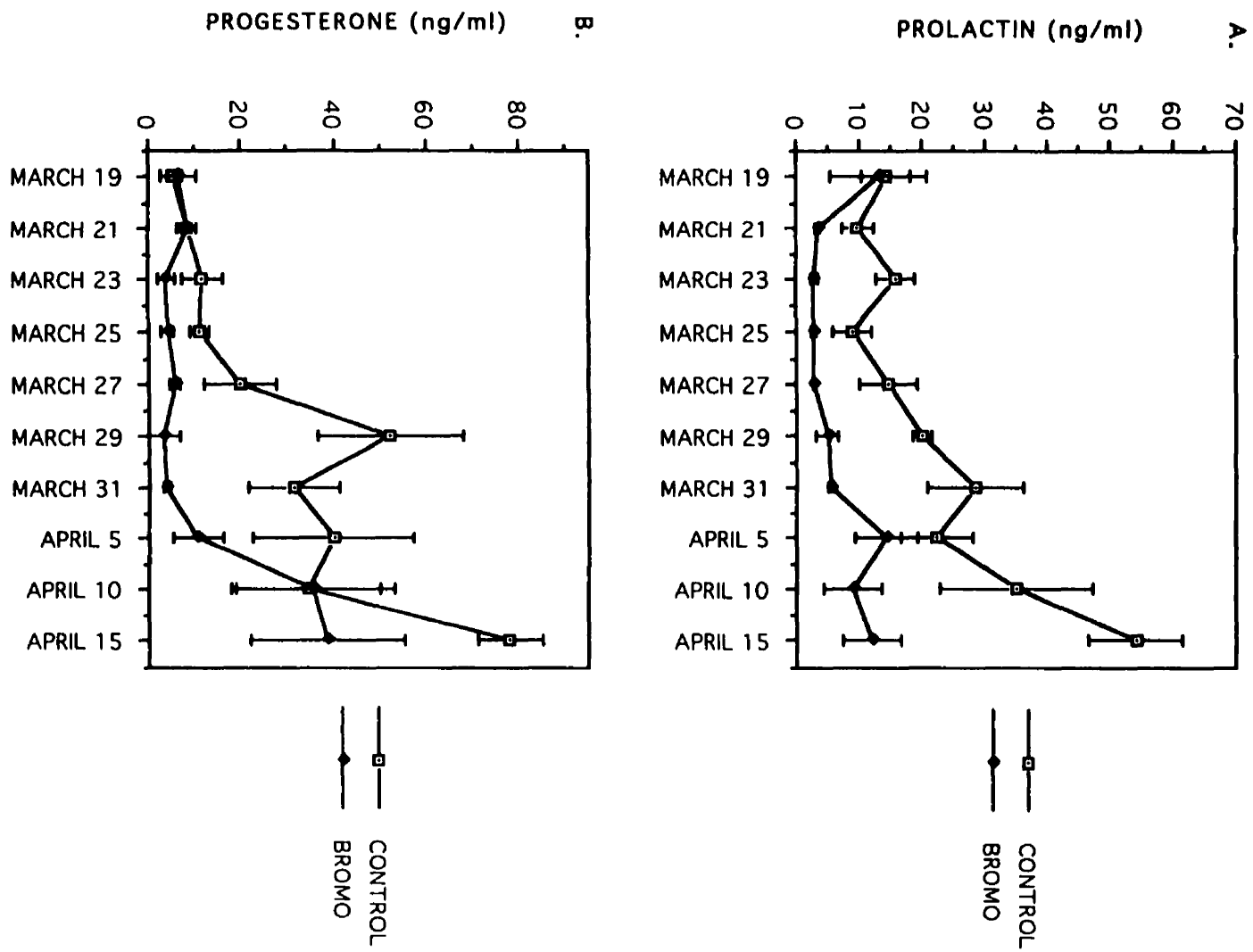

PROLACTIN (ng/ml)
A.
0
10
20
30
40
50
60
70
MARCH 19
MARCH 21
MARCH 23
MARCH 25
MARCH 27
MARCH 29
MARCH 31
APRIL 5
APRIL 10
APRIL 15
CONTROL
BROMO
PROGESTERONE (ng/ml)
B.
0
20
40
60
80
MARCH 19
MARCH 21
MARCH 23
MARCH 25
MARCH 27
MARCH 29
MARCH 31
APRIL 5
APRIL 10
APRIL 15
CONTROL
BROMO

Figure 5. (a). Mean (+ SEM) of PRLr mRNA abundance in ovarian tissue collected from pregnant mink every 3 days between March 19 and March 31 and every 5 days there after until April 15. Solid boxes represent animals treated with 2 mg/day of bromocryptine. Open boxes represent control animals. (b). Top panel - a representative control Northern blot hybridized with the mink PRLr probe. Lower panel - the same control Northern blot rehybridized with a ribosomal 28S probe. (c). Top panel - a representative bromocryptine Northern blot hybridized with the mink PRLr probe. Lower panel - the same Northern blot rehybridized with a human ribosomal 28S probe.

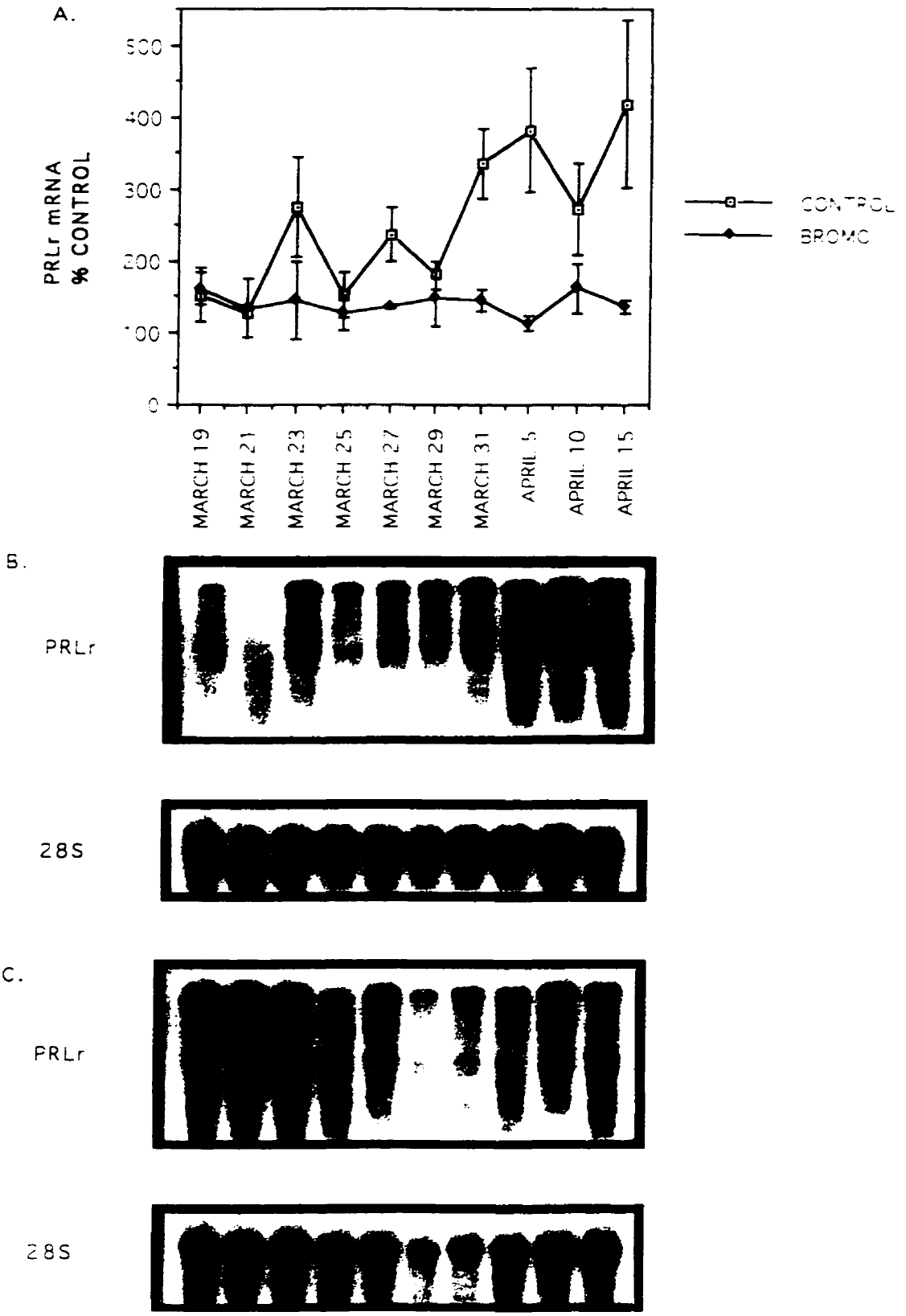

A.
PRLr mRNA
% CONTROL
500
400
300
200
100
0
MARCH 19
MARCH 21
MARCH 23
MARCH 25
MARCH 27
MARCH 29
MARCH 31
APRIL 5
APRIL 10
APRIL 15
CONTROL
BROMO
B.
PRLr
28S
C.
PRLr
28S

Figure 6. Mean (+ SEM) of prolactin receptor binding in pregnant mink ovaries collected between March 19 and April 15. Results are expressed as the number of counts per mg of protein.

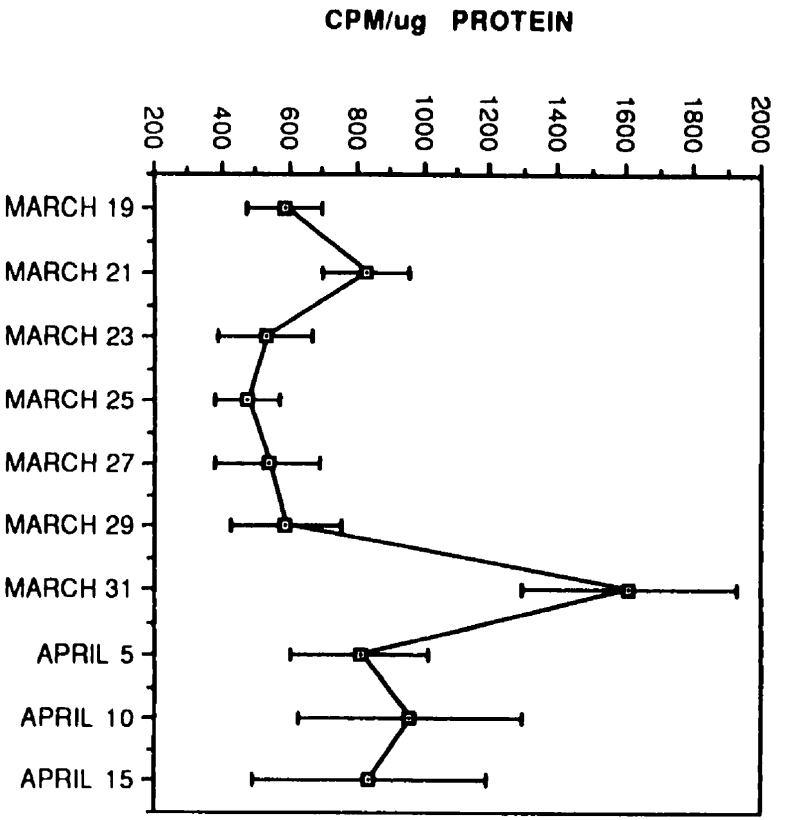
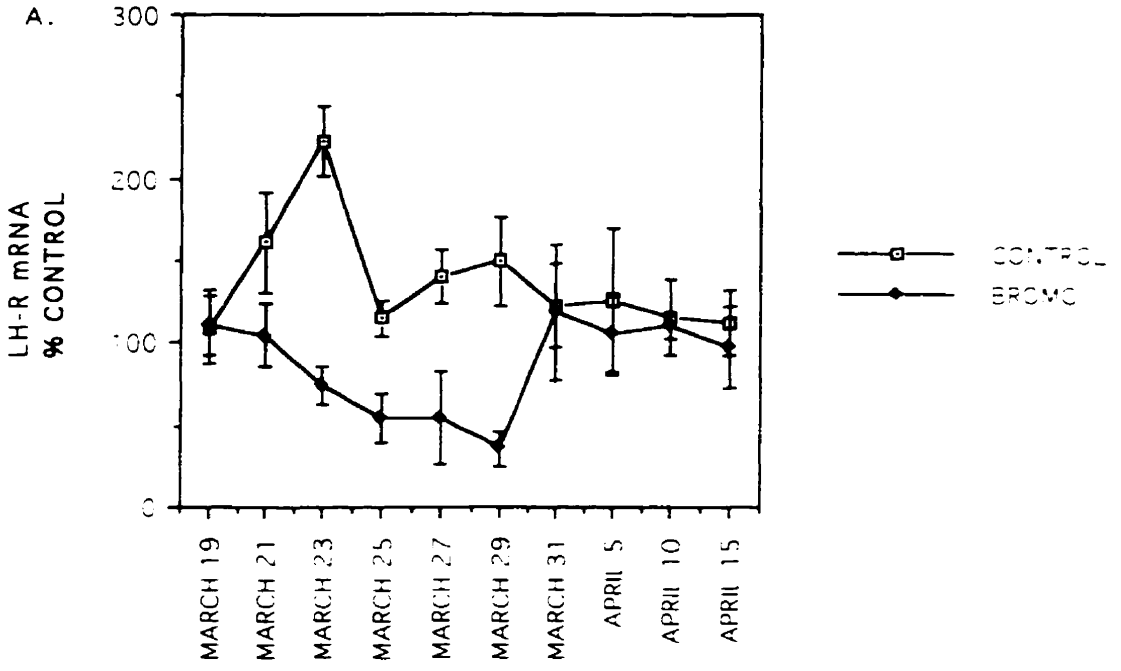

CPM/ug PROTEIN
200
400
600
800
1000
1200
1400
1600
1800
2000
MARCH 19
MARCH 21
MARCH 23
MARCH 25
MARCH 27
MARCH 29
MARCH 31
APRIL 5
APRIL 10
APRIL 15

Figure 7. (a). Mean (+ SEM) of LHr mRNA abundance in ovarian tissue collected from pregnant mink every 3 days between March 19 and March 31 and every 5 days there after until April 15. Solid boxes represent animals treated with 2 mg/day of bromocryptine. Open boxes represent control animals. (b). Top panel - a representative control Northern blot hybridized with the mink LHr probe. Lower panel - the same representative bromocryptine Northern blot hybridized with the mink LHr probe. Lower panel - the same Northern blot rehybridized with a human ribosomal 28S probe.


A.
300
200
100
0
LH-R mRNA
% CONTROL
MARCH 19
MARCH 21
MARCH 23
MARCH 25
MARCH 27
MARCH 29
MARCH 31
APRIL 5
APRIL 10
APRIL 15
CONTROL
BROMO

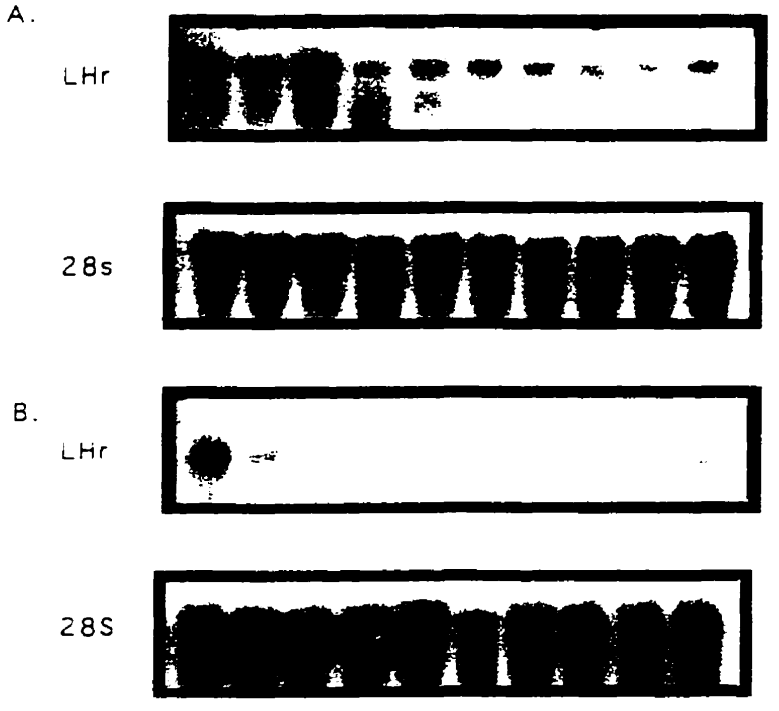

A.
LHr
28s
B.
LHr
28S

Table 1. Nucleotide (nt) and amino acid (aa) sequence homology comparison for mink PRLr and LHr genes with published information in other species.

	PRLr		LHr	
	(nt %)	(aa %)	(nt %)	(aa %)
BOVINE	84.0	77.8	89.8	92.5
HUMAN	81.4	73.3	90.5	92.8
MURINE	79.1	72.4	84.3	87.6
RABBIT	85.6	84.8	NA	NA
PORCINE	NA	NA	89.5	91.6
RAT	78.4	72.4	84.7	89.4

3.6 DISCUSSION

A significant portion of the extra-cellular and all of the transmembrane domain of the mink prolactin receptor gene was cloned and sequenced. It displayed high sequence homology with the rat (Boutin *et al.*, 1988, Shirota *et al.*, 1990), murine (Davis and Linzer, 1989), human (Boutin *et al.*, 1989), bovine (Scott *et al.*, 1992) and rabbit (Edery *et al.*, 1989) PRLr genes. It also displayed 53-54% homology with the rat (Mathews *et al.*, 1989), human (Leung *et al.*, 1987) and rabbit (Leung *et al.*, 1987) and growth hormone receptor gene which is part of the same GH/PRL/cytokine receptor superfamily (Kelly *et al.*, 1991). In the portion of the extra-cellular domain cloned, there were 4 cysteine residues present. These residues have been conserved in all PRLr sequences cloned to date. In addition, there was another conserved cysteine residue present in the transmembrane domain. The prolactin receptor is believed to be a glycosylated protein (Kelly *et al.*, 1991). Two potential N-linked glycosylation sites were found in the extra-cellular domain of the mink PRLr gene fragment. The current experiment did not determine whether the mink fragment was of the long or short form of the PRLr, since differences in PRLr type are due to alterations in the cytoplasmic region of the gene (Davis and Linzer, 1989; Shirota *et al.*, 1990), which was not amplified in the present study.

Northern blot analysis revealed that as with other species, the mink PRLr probe hybridized with multiple transcripts. In the case of the mink these were approximately 3.4, 4.4 and 10.5 kb. The long form of the rat PRLr is encoded by three transcripts 2.5, 3.0 and 5.5 kb (Shirota *et al.*, 1990) while the rabbit PRLr is encoded by 3 major transcripts which migrated at 2.7, 3.4 and 10.5 kb and one minor transcript at 6.2 kb (Dusanter-Fourt *et al.*, 1991). The human PRLr is encoded by three transcripts which are 2.5, 3.0 and 7.3 kb in size (Boutin *et al.*, 1989).

In the present study tissue distribution analysis revealed a strong PRLr hybridization signal with mink ovary, CL and testes. Longer exposure of the blot revealed weak signals are present in the uterus, adrenal, kidney and liver. The wide tissue distribution of PRLr mRNA in the current experiment and reported by others (reviewed by Kelly *et al.*, 1991) is not unexpected since prolactin has a wide spectrum of activities which includes effects on 1) reproduction and lactation, 2) water and salt balance, 3) growth and morphogenesis, 4) metabolism, 5) behavior, 6) immunoregulation and 7) the ectoderm and skin (Nicoll and Bern, 1972).

Prolactin receptor in the mink ovary is of physiological importance because of the role of prolactin as the principal luteotropic hormone in this species (Papke *et al.*, 1980, Martinet *et al.*, 1981, Murphy *et al.*, 1980 and 1981). The current experiment demonstrated that the abundance of PRLr mRNA and receptor binding was low during the

delay phase of gestation and did not increase significantly during the early stages of CL reactivation. However, prolactin levels and PRLr expression were of sufficient magnitude at that time to invoke the termination of embryonic diapause and reactivation of the CL, as indicated by the increase in progesterone secretion seen on March 25. Treatment of animals with bromocryptine did not affect basal levels of PRLr mRNA; however, it prevented the pre-implantation rise in serum prolactin and PRLr mRNA and thus prevented the reactivation of the mink CL and progesterone production. The abundance of PRLr mRNA and the number of prolactin binding sites increased dramatically after March 29 reaching their highest levels during implantation and early post-implantation gestation, the time when progesterone production is at its highest. Treatment of animals with bromocryptine completely abolished this increase in PRLr mRNA but did not decrease PRLr mRNA levels below pre-treatment values. Since this study revealed that PRLr mRNA and receptor binding were closely correlated to serum prolactin levels, it is reasonable to conclude that in the mink prolactin regulates its own receptor, as has been shown in a variety of tissues and species. An inductive effect of prolactin was first postulated when it was observed that a renal capsular pituitary implant in hypophysectomized rats augmented hepatic PRLr approximately 48 hours after an increase in serum PRL (Posner *et al.*, 1975). PRL secreting tumors were also found to be associated with elevated hepatic receptor levels (Posner, 1976). Prolactin induction of its own receptor has also been demonstrated in rat lung (Amit *et al.*, 1985), liver (Amit *et al.*, 1985; Manni *et al.*, 1978; Barash *et al.*, 1988), and kidney (Barash *et al.*, 1986) as well as in the liver of the Snell-dwarf mouse (Knazek *et al.*, 1977) and the testes of the golden hamster (Klemcke *et al.*, 1984; Klemcke *et al.*, 1986).

The putative transmembrane and cytoplasmic region of the mink LHr gene was amplified and sequenced. It had high nucleic acid sequence homology with the rat (McFarland *et al.*, 1989), porcine (Loosfelt *et al.*, 1989), human (Frazier *et al.*, 1990; Minegishi *et al.*, 1990) and murine (Gudermann *et al.*, 1992) LHr gene sequences. Of 22 potential phosphorylation sites (serine, threonine, tyrosine residues) in the intra-cellular region of rat LHr (Segaloff and Ascoli, 1993), 19 were conserved in the deduced amino acid sequence of the mink LHr. The number and location of potential phosphorylation sites in the first and second intracellular loops were the same in the mink and rat. However, in the third intra-cellular loop one of the three potential phosphorylation sites in the rat was shifted from a threonine to a methionine in the mink. Hipkin *et al.* (1995) provided evidence which indicated that serine residues at amino acid positions 635, 639, 649 and 652 of the LH/CG receptor were phosphate acceptors in response to human chorionic gonadotropin (hCG) and phorbol 12-myristate-13-acetate (PMA). In the mink, however, only two of these serine residues were conserved (639 and 652). In addition to the 19

potential phosphorylation sites conserved between the rat and mink, a further six sites were present in the cytoplasmic tail of the mink LHr. Segaloff and Ascoli (1993) reported that 20 cysteine residues were conserved among the four species studied. Eleven of these residues were present in the transmembrane and cytoplasmic regions of the receptor. All cysteine residues were conserved in the deduced amino acid sequence of the mink LHr, including two cysteine residues at amino acid position 621 and 622 of the rat LHr, which are believed to be palmitoylated (Zhu *et al.*, 1995).

Using the homologous mink LHr cDNA fragment as a probe, multiple LHr mRNA transcripts were identified. Multiple transcripts have been discerned in a number of other species including the rat (Hu *et al.*, 1990; LaPolt *et al.*, 1990, Wang *et al.*, 1991), mouse (Wang *et al.*, 1991), human (Minegishi *et al.*, 1990), pig (Loosfelt *et al.*, 1989) and sheep (Bacich *et al.*, 1994). The number, size and abundance of these transcripts vary among species. In addition, these parameters vary among different tissue types (reviewed by Segaloff and Ascoli, 1993). The principal transcript identified in the mink CL was 2.4 kb, large enough to encode the entire LHr protein, since it has an open reading frame of approximately 2 kb (Segaloff and Ascoli, 1993). In the current experiment, the mink LHr probe hybridized only to transcripts in the ovary, CL and testes. No LHr message was detected in the brain or the uterus in contrast to reports in other species (Lei *et al.*, 1993; Bonnamy *et al.*, 1993).

The abundance of LHr mRNA varied significantly over the pre-implantation and early post-implantation stages of mink gestation, in a manner which was strikingly different from the pattern for PRLr. Levels of LHr mRNA increased dramatically between March 19 and 23, but fell by March 25. This transient peak in LHr mRNA occurred during the critical period of CL reactivation and directly preceded the pre-implantation increase in progesterone production, providing indirect evidence for a role for LH in the reactivation of the mink CL. This contradicts previous studies in which it was suggested that LH has little involvement in the reactivation of the mink CL. In hypophysectomized mink, prolactin alone was able to induce CL reactivation as indicated by an increase in peripheral progesterone concentrations (Murphy *et al.*, 1981). However, this study also demonstrated that prolactin treatment alone was unable to maintain progesterone production and that another factor or factors may be necessary. A further study suggests that LH and follicle-stimulating hormone (FSH) were not required for luteal support during embryonic diapause or for luteal reactivation, since passive immunization of animals with monoclonal antibodies against gonadotropin releasing hormone (GnRH) had no effect on serum progesterone levels during pre-implantation gestation (Murphy *et al.*, 1993). However, it was not conclusively shown that LH and FSH levels were completely suppressed by this treatment in these animals.

In the current experiment, treatment of animals with bromocryptine prevented the transient LHr mRNA peak between March 19 and 25 and reduced LHr mRNA levels below those of the pre-treatment controls. Thus the role for prolactin in the reactivation and subsequent function of the mink CL may involve the regulation and maintenance of LH receptors. This hypothesis is supported by a number of studies which have shown that prolactin and/or placental lactogens are involved in the regulation of LHr binding sites (Gibori and Richards, 1978; Jammes *et al.*, 1985) and LHr mRNA (Gafvels *et al.*, 1992; Bjurulf *et al.*, 1994) in the CL of other species.

In conclusion, fragments of the mink PRLr and LHr genes were cloned and sequenced and had high sequence homology with other species. The abundance of mRNA for both genes varied significantly over the course of CL reactivation and early post-implantation gestation. The pattern of expression for both of these genes was greatly influenced by endogenous prolactin levels indicating a role for prolactin in the regulation and maintenance of these receptors in the mink ovary.

3.7 ACKNOWLEDGMENTS

We would like to thank Drs. D. Zadworny and D. Silversides for providing us with some of the PCR primers utilized in these experiments. We would also like to thank Mr. M. Morrow for animal care. Technical assistance was provided by Mrs. M. Dobias Goff. This project was supported by MRC and NSERC grants to BDM.

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3.9 STATEMENT OF PARTICIPATION

Experimental Design - Deborah Douglas, Bruce Murphy

Animal Handling - Deborah Douglas, Jianhua Song

Sample Collection - Deborah Douglas, Jianhua Song

Cloning and Sequencing - Deborah Douglas, Alan Houde

Northern and Slot Blot Analyses - Deborah Douglas

Progesterone Assay - Jianhua Song

Prolactin Assay - Pat Concannon

Statistical Analysis - Deborah Douglas

Interpretation of Results - Deborah Douglas, Bruce Murphy

Paper - Deborah Douglas

3.10 CONNECTOR

In Paper #1, the objective was to study changes in the abundance of PRLr and LHr mRNA over the course of mink embryonic diapause, CL reactivation and early post-implantation gestation, and to determine prolactins effects on these luteotropic hormone receptors. In this model, pregnant animals were exosed to endogenous prolactin levels or had their prolactin levels suppressed through the administration of the dopamine agonist bromocryptine. In Paper #2, the same whole animal model was used to study changes in the abundance of mRNA for the steroidogenic elements P450scc, 3β-HSD and StAR, to determine if prolactin exerts its luteotropic effects on progesterone production through any of these elements.

THE OCCURRENCE OF P450 SIDE CHAIN CLEAVAGE (P450scc), 3β-HYDROXYSTEROID DEHYDROGENASE (3β-HSD) AND STEROIDOGENIC ACUTE REGULATORY PROTEIN (StAR) mRNA IN THE MINK (*Mustela vison*) OVARY THROUGH GESTATION.

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Key Words: Mink, embryonic diapause, prolactin, P450scc, 3β-HSD, StAR.

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4.2 ABSTRACT

During embryonic diapause the mink corpus luteum (CL) produces low levels of progesterone. After the vernal equinox the CL is reactivated and begins to produce progesterone in response to prolactin. The objective of this study was to monitor changes in the abundance of mRNA for P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD) and steroidogenic acute regulatory protein (StAR), through mink gestation. Corpora lutea were collected from three mink every five days, during the pre-implantation stage of mink gestation (March 21 to April 10) and every five days during the post-implantation gestation (April 10 to April 25). To determine the effects of prolactin on these parameters, ovaries were collected every three days from two groups of animals between March 19 and April 15. The first group was exposed to endogenous levels of prolactin, while the second had prolactin levels suppressed by continuous administration of 2-bromo-α-ergocryptine (bromocryptine). Northern blot analysis revealed that both a porcine P450scc and a human 3β-HSD probe hybridized to single mink mRNA transcripts 1.9 and 2.0 kb, respectively, while a mouse StAR probe hybridized to two transcripts 1.9 and 3.9 kb. In all cases, hybridization was specific to steroidogenic tissue (ovary, testes and adrenal). The abundance of P450scc mRNA did not vary over the pre-implantation stage of mink gestation but decreased by a mean value of 60% during post-implantation gestation. Suppression of endogenous prolactin levels by bromocryptine did not alter P450scc mRNA levels. The abundance of 3β-HSD mRNA increased (2.5 fold) during the pre-implantation stage of gestation, however, during the post-implantation gestation, 3β-HSD mRNA levels fell by half as parturition approached. Suppression of endogenous prolactin prevented the pre-implantation increase in 3β-HSD mRNA abundance. The abundance of StAR mRNA did not vary significantly over the experimental period and bromocryptine induced alterations in serum prolactin levels had no significant effect ($P<0.05$). In conclusion, reactivation of the mink corpus luteum and the subsequent increases in progesterone secretion does not appear to involve alteration in the abundance of P450scc or StAR mRNA. However, the abundance of 3β-HSD mRNA was highly correlated with progesterone production and the pre-implantation increase in 3β-HSD mRNA could be prevented by suppressing prolactin levels. Prolactin may induce CL reactivation and stimulate progesterone production in the mink by means of the regulation of transcription of the 3β-HSD gene.

4.3 INTRODUCTION

The annual reproductive cycle in the mink is characterized by an obligatory period of embryonic diapause or a delay in implantation (Hansson, 1947; Enders, 1952). The length of the delay period is relatively short and persists only a few weeks, compared to several months as seen in other mustelids (Mead, 1981). In this species, ovulation is induced within 48 hours by copulation (Hansson, 1947; Enders, 1952). After follicle rupture the CL undergoes a short proliferative phase, during which time it goes from a hollow ball to a solid mass of tissue (Hansson, 1947; Douglas *et al.*, 1994). The CL then becomes inactive and undergoes some degree of involution (Douglas *et al.*, 1994). During this time it produces low levels of progesterone (Moller, 1973) and it is only after the vernal equinox on March 21, when prolactin levels are increasing, that the CL is reactivated and begins to produce progesterone. Some progress has been made in determining of the hypophyseal mechanisms by which embryonic diapause is terminated in this species. Prolactin acts directly to reactivate the CL since exogenous prolactin will advance the timing of implantation in the intact mink (Papke *et al.*, 1980; Martinet *et al.*, 1981) and prolactin alone can induce implantation in the hypophysectomized mink (Murphy *et al.*, 1981). It is presumed that prolactin exerts its effects directly on the CL.

The steroidogenic pathway by which progesterone is synthesized has been well documented. The principal enzymes involved in luteal progesterone production are cytochrome P450scc and 3β-HSD. Cytochrome P450scc, along with its electron donors adrenodoxin and adrenodoxin reductase are responsible for the 20-hydroxylation, 22-hydroxylation and cleavage of the C20-C22 bond of cholesterol to produce pregnenolone and isocaproic acid (Miller, 1987, and 1988). Subsequently the oxidation and isomeration of the Δ^5-3β-hydroxysteroid pregnenolone into the Δ^4-3-ketosteroid progesterone is carried out by 3β-HSD (Luu-The *et al.*, 1989; Lorence *et al.*, 1990).

The amount of progesterone produced by the CL or ovary is not only dependent on the activities of specific steroidogenic enzymes but also upon the provision of cholesterol as substrate from *de novo* synthesis, cholesterol ester stores and low density lipoproteins (LDL: reviewed by Murphy and Silavin, 1989). Waterman (1995) reviewed information which indicates that mobilization of cholesterol from lipid stores to the vicinity of P450scc in the inner mitochondrial membrane is essential in ligand regulated steroid biosynthesis. The mitochondrial protein StAR, recently characterized by Clark *et al.* (1994), has been proposed to be the cholesterol transport protein.

The nucleic acid and deduced amino acid sequences for the genes encoding P450scc, 3β-HSD and StAR have been determined for a number of different species (P450scc; bovine: Morohashi *et al.*, 1984; human: Chung *et al.*, 1986; rat: Oonk *et al.*,

1989; porcine: Mulheron *et al.*, 1989), (3β-HSD; human placenta: Luu-The *et al.*, 1989; bovine: Zhao *et al.*, 1989; rat liver: Zhao *et al.*, 1990; rat : Zhao *et al.*, 1991; macaque: Simard *et al.*, 1991; mouse: Bain *et al.*, 1991), (StAR; mouse: Clark *et al.*, 1994; human: Sugawara *et al.*, 1995; sheep; Juengel *et al.*, 1995). Thus, probes are available to determine changes in the abundance of mRNA for these genes in different tissues under various experimental conditions. The objective of this study was to determine the changes in levels of mRNA for P450scc, 3β-HSD and StAR over the course of mink gestation. Given the unique dependence of the carnivore CL on prolactin, it was of interest to determine whether the mRNA levels of these genes can be correlated with changes in serum prolactin and progesterone, as well as with CL reactivation and embryo implantation.

4.4 MATERIALS AND METHODS

4.4.1 EXPERIMENTAL DESIGN

Adult female mink were maintained on a commercial mink ranch (Morrow Fourrures; St Paul d'Abbotsford, PQ). They received a standard wet mink ration and water *ad libidum*. Beginning on March 3 all female mink were exposed to males every two days until mating. Seven to nine days later, females were remated to different males. Successful matings were determined by the presence of motile sperm in vaginal smears.

In the first trial ovaries were collected from three pregnant mink at intervals of five days between March 21 and April 5. Animals were anesthetized using ketamine hydrochloride (0.3 ml/animal, Rogar/STB, Montreal, PQ) and a terminal blood sample was collected via cardiac puncture. The animals were then sacrificed using T61 (0.5-1.0 ml/animal, Hoechst, Regina, SK) and the ovaries plus various tissue samples (liver, kidney, adrenal, uterus) were collected. Corpora lutea were excised from the surrounding interstitial tissue under a dissecting microscope. All samples were immediately placed in 4 M guanidinium isothiocyanate (GITC: Gibco/BRL, Burlington, ON) plus 0.12 M of 2-mercaptoethanol (Sigma, St Louis, MO.), snap frozen in liquid nitrogen and stored at -70 C until total RNA was extracted. Corpora lutea collected during the post-implantation stage of gestation followed a similar protocol except ovaries were collected at 5 day intervals between April 10 and April 25.

In order to determine the effects of prolactin on the abundance of P450scc, 3β-HSD and StAR mRNA a second experiment was conducted. Whole ovaries and a terminal blood sample were collected from pregnant mink every three days beginning March 19 until March 31 and every five days thereafter until April 15. The animals were divided into two treatment groups. All of the animals were implanted with Alzet osmotic minipumps (Alza Corp., Palo Alto, CA) on March 20. The first group received pumps which released 2

mg/day of the dopamine agonist bromocryptine (Sigma), while the second group received pumps which contained saline and acted as controls.

4.4.2 NORTHERN AND SLOT BLOT ANALYSES

Total RNA was extracted by CsCl (Gibco/BRL) gradient ultracentrifugation as described by Chirgwin *et al.* (1979). The samples were thawed and homogenized with a PT 3000 polytron (Brinkmann, Rexdale, ON). The homogenate was then layered onto a 5.7 M CsCl gradient and centrifuged at 32,000 rpm (174,000g) using a SW-41 rotor (Beckman, Mississauga, ON), for 20 hours at 22 C. The RNA pellet was dissolved and precipitated twice in 0.1 volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol. The pellet was redissolved in diethyl pyrocarbonate (DEPC: Sigma)-treated distilled water and stored at -70 C. Nucleic acid concentrations for each sample were determined by spectrophotometric measurement at an absorbance of 260 nm.

For Northern analysis, 15 µg of total RNA was size fractionated on a 1% agarose denaturing formaldehyde gel and stained with ethidium bromide (Sigma) as described by Sambrook *et al.* (1989). The RNA was transferred by capillary action overnight using 20 x SSC (1 x SSC = 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) onto a nylon membrane (Hybond-N: Amersham, Oakville, ON). The RNA was cross-linked to the membrane using GS gene linker UV chamber (BioRad, Mississauga, ON).

For slot blot analysis, 6 µg of total RNA in 50 µl of DEPC-treated water was heated to 65 C for 10 minutes with 150 µl of denaturing solution (98 µl de-ionized formamide, 32 µl 37% formaldehyde and 20 µl of 10 x MOPS (0.2 M 3-[N-morpholino] propanesulphonic acid, 0.5 M sodium acetate, pH 7.0, 0.01 M sodium EDTA)). An equal volume of ice-cold 20 x SCC was added to the samples, which were applied to a nylon membrane using a slot blot manifold (BioRad).

Prior to hybridization the membranes were rinsed in 5 x SSPE (1 x SSPE = 180 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) then prehybridized for 30 minutes at 60 C in hybridizing solution (5 x SSPE, 5 x Denhardt's solution: 0.5 % sodium dodecyl sulfate (SDS), 10% dextran sulfate). Denatured herring sperm DNA (10 mg/ml: Sigma) was added to the hybridizing solution and the pre-hybridization period continued for a minimum of 3 hours. Complementary DNA probes (porcine P450scc: Mulheron *et al.*, 1989; human 3β-HSD: Luu-The *et al.*, 1989; mouse StAR: Clark *et al.*, 1994) were labeled by random primer extension (Boehringer Mannheim, Laval, PQ) with [^{32}P]-dCTP (Dupont, Mississauga, ON). Once labeled, probes were denatured and added to the hybridizing solution. The incubation was continued overnight at 60 C. The following day the membranes were washed twice, 15 minutes per wash, with 2 x SSPE plus 0.1% SDS at room temperature and twice at 60 C. The membranes were sealed in hybridizing bags with

a small amount of 5 x SSPE and exposed to Kodak XAR-5 film (Rochester, NY) in cassettes with intensifying screens. All membranes were rehybridized with a human ribosomal 28S probe (Gonzales *et al.*, 1985). Autoradiographic images were digitized and analyzed using Collage software. Results are expressed as a ratio between the cDNA probe of interest and ribosomal 28S. These arbitrary units were then expressed as a percentage of a control sample derived from a pool of ovaries from anestrous animals which was present on all of the Northern and slot blots.

4.4.3 HORMONE ASSAYS

Mink serum progesterone concentrations were determined by liquid-phase radioimmunoassay after extraction in 10 volumes of hexane (BDH, Darmstadt). Extraction recoveries ranged between 95-98%. An antiserum raised against 4-pregnen-11α-o-13,20dione hemisuccinate bovine serum albumin, kindly provided by Dr. A Goff (Lafrance and Goff, 1985) was used as the first antibody. Progesterone-11α-glucuronide-[^{125}I] iodotryramine (Amersham, Oakville, ON) was used as radioactive tracer and goat anti-rabbit IgG (Prince Laboratories, Toronto, ON) as the precipitating second antibody. The sensitivity of the assay was 6 pg.

Mink serum prolactin concentrations were determined by a double antibody radioimmunoassay, previously validated for the mink (Murphy *et al.*, 1990). The sensitivity of the assay was determined to be 0.3 ng.

4.4.4 STATISTICAL ANALYSIS

Mean (+ SEM) values were calculated for each parameter. One way analysis of variance was employed to determine significant differences over the experimental periods in both the pre and post-implantation experiments. Two way analysis of variance was used to determine differences between the bromocryptine and control groups over the experimental period. In the presence of a significant F value, individual comparisons were made by the LSD method. A value of $P<0.05$ was considered significant.

4.5 RESULTS

Northern analysis of the tissue distribution blots revealed that the porcine P450scc and human 3β-HSD probes bound to single mink transcripts of 1.9 and 2.0 kb in size, respectively. Further, transcripts for P450scc (Figure 1a) and 3β-HSD (Figure 1b) were exclusively located in steroidogenic tissue (ovary, testes and adrenal). The StAR probe (Figure 1c) hybridized to two transcripts 1.9 and 3.9 kb in size and was found exclusively in steroidogenic tissue. Densitometric scanning revealed that although the larger transcript

predominated the two transcripts did not appear to be differentially regulated (data not shown).

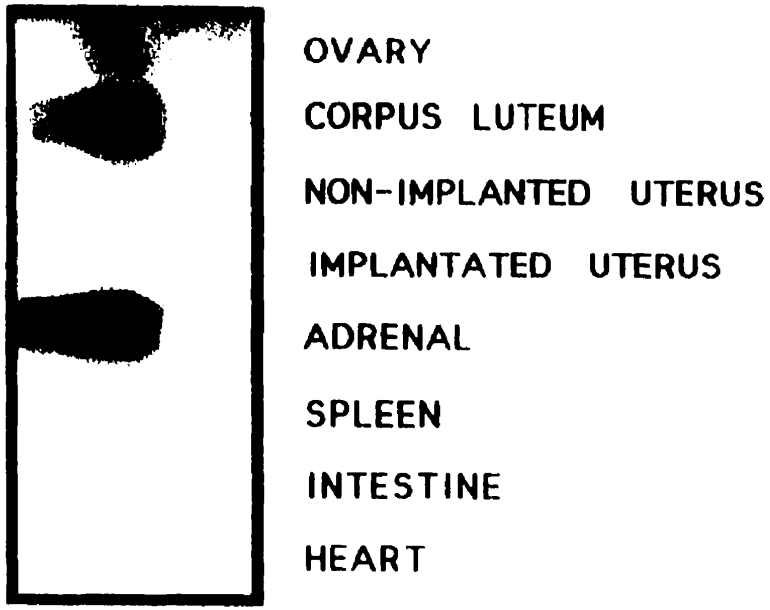
Analysis of the first trial revealed that during the pre-implantation stage of mink gestation at the time of CL reactivation (March 21 to April 5) serum progesterone levels increased significantly ($P<0.05$) from a mean of 7.7 + 0.9 to 83.7 + 14.5 ng/ml (Figure 2b). Over the same period there was no change in the abundance of luteal P450scc mRNA (Figure 3a). However, the abundance of mRNA for 3β-HSD (Figure 4a) increased 2.5 fold and was found to be positively correlated with serum progesterone levels ($P<0.05$, $r=0.55$). Serum progesterone levels (Figure 5b) peaked at a mean value of 87.7 + 14.5 ng/ml and approximately to the time of embryo implantation. The levels then decreased gradually to a mean value of 28.7 + 6.4 ng/ml shortly before parturition. During post-implantation gestation, the abundance of P450scc mRNA (Figure 6) decreased by 60% ($P<0.05$). The abundance of 3β-HSD mRNA (Figure 7) also decreased over this same time period (44.0%, $P<0.05$).

Treatment of pregnant mink with bromocryptine prevented the increase in serum prolactin levels normally associated with the vernal equinox in this species (Figure 8a). As a result of prolactin suppression, serum progesterone levels were also suppressed (Figure 8b). However, several animals near the end of the experiment (April 5) appeared to have escaped the inhibitory effects of bromocryptine since they showed increases in both serum prolactin and progesterone levels.

Neither the abundance of P450scc nor StAR mRNA varied significantly between March 19 and April 15 and neither was affected by suppression of endogenous prolactin (Figure 9a and 9b). As in the first experiment, the abundance of 3β-HSD mRNA increased (3.5 fold) during pre-implantation gestation peaking on April 15. Suppression of prolactin with bromocryptine prevented this increase (Figure 9c).

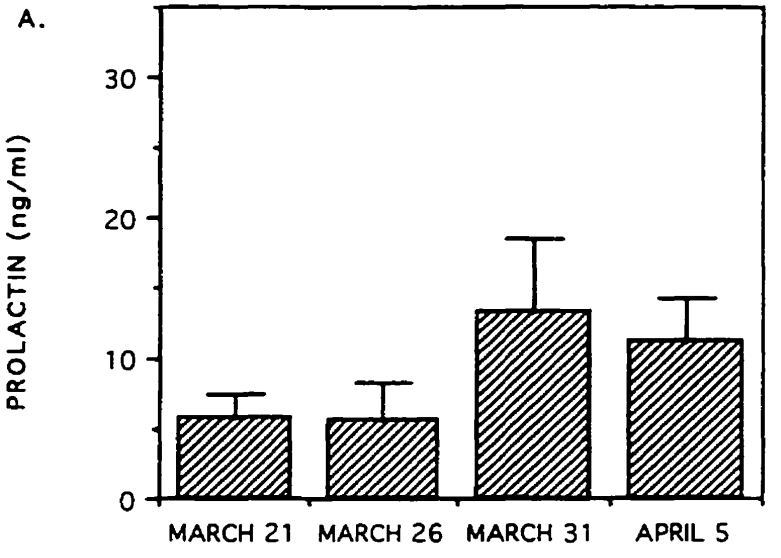
Figure 1. (a). Northern blot showing the tissue distribution of P450scc mRNA in the mink. The heterologous porcine P450scc (Mulheron *et al.*, 1989) probe hybridized with a single mink transcript 1.9 kb in size. (b). Northern blot showing the tissue distribution of 3β-HSD mRNA in the mink. The heterologous human 3β-HSD probe (Luu-The *et al.*, 1989) hybridized with a single mink transcript 2.0 kb in size. (c). Northern blot showing the tissue distribution of StAR mRNA in the mink. The heterologous mouse StAR probe (Clark *et al.*, 1994) hybridized with two transcripts 1.9 and 3.8 kb in size.

A
1.9 kB
OVARY
CORPUS LUTEUM
NON-IMPLANTED UTERUS
IMPLANTATED UTERUS
ADRENAL
SPLEEN
INTESTINE
HEART


B
2.0 kB
OVARY
CORPUS LUTEUM
NON-IMPLANTED UTERUS
IMPLANTATED UTERUS
ADRENAL
SPLEEN
INTESTINE
HEART

OVARY
CORPUS LUTEUM
TESTES
ADRENAL
LIVER
KIDNEY
INTESTINE
HEART
C
3.9 kB
1.9 kB

Figure 2. Mean (+ SEM) of serum (a). prolactin, and (b). progesterone concentrations in pregnant mink during the pre-implantation stage (March 21 to April 5) of gestation. Prolactin and progesterone levels were determined by double antibody RIA.


A.
PROLACTIN (ng/ml)
30
20
10
0
MARCH 21
MARCH 26
MARCH 31
APRIL 5

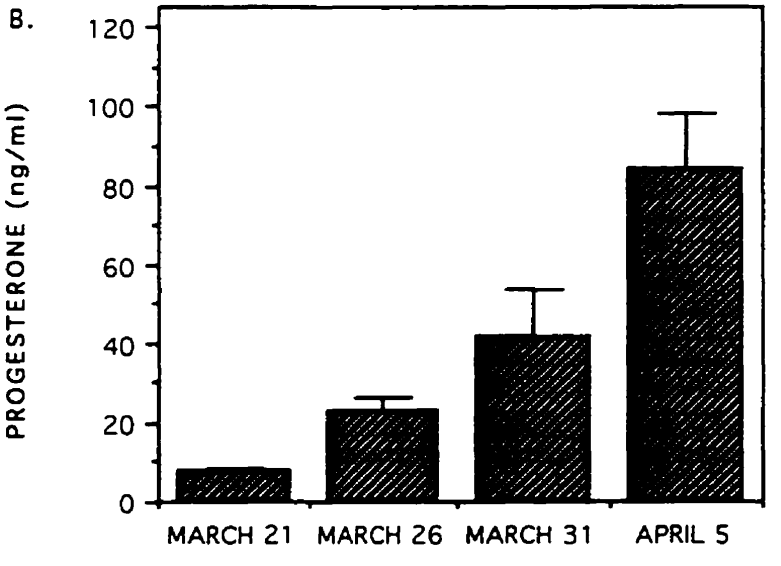
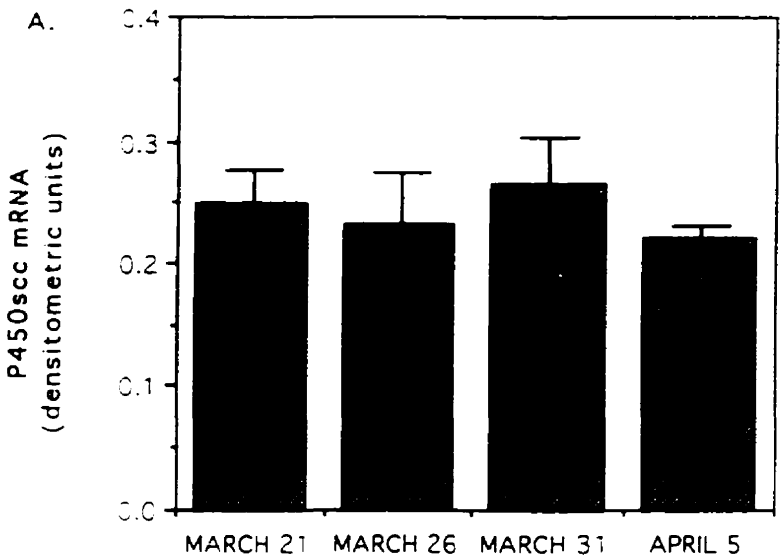
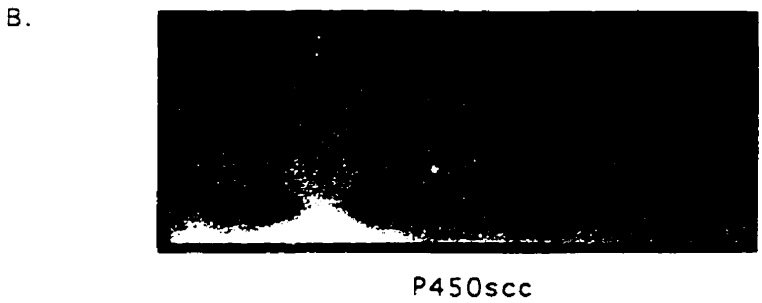

B.
PROGESTERONE (ng/ml)
120
100
80
60
40
20
0
MARCH 21
MARCH 26
MARCH 31
APRIL 5

Figure 3. (a). Abundance of P450scc mRNA (mean + SEM) in pregnant mink CL during the pre-implantation stage (March 21 to April 5) of gestation. Abundance of mRNA for P450scc was determined by slot blot analysis using 28S as a constitutive control. P450scc mRNA levels are expressed in arbitrary densitometric units. (b). Autoradiograph of P450scc labeled slot blot. (c). Autoradiograph of human ribosomal 28S labeled slot blot.


A.
P450scc mRNA
(densitometric units)
0.4
0.3
0.2
0.1
0.0
MARCH 21
MARCH 26
MARCH 31
APRIL 5


B.
P450scc

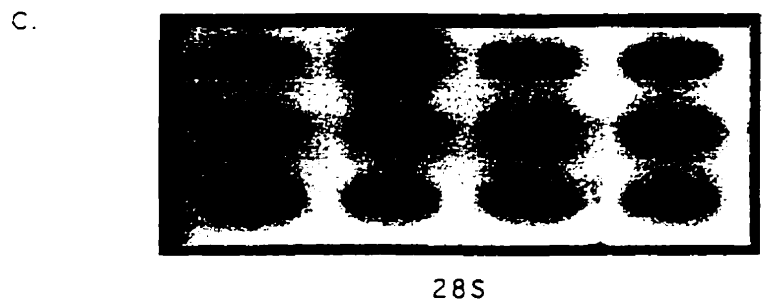

C.
28S

Figure 4. (a). Abundance of 3β-HSD mRNA (mean + SEM) in pregnant mink CL during the pre-implantation stage (March 21 to April 5) of gestation. Abundance of mRNA for 3β-HSD was determined by slot blot analysis using 28S as a constitutive control. 3β-HSD mRNA levels are expressed in arbitrary densitometric units. (b). Autoradiograph of 3β-HSD labeled slot blot. (c). Autoradiograph of human ribosomal 28S labeled slot blot.

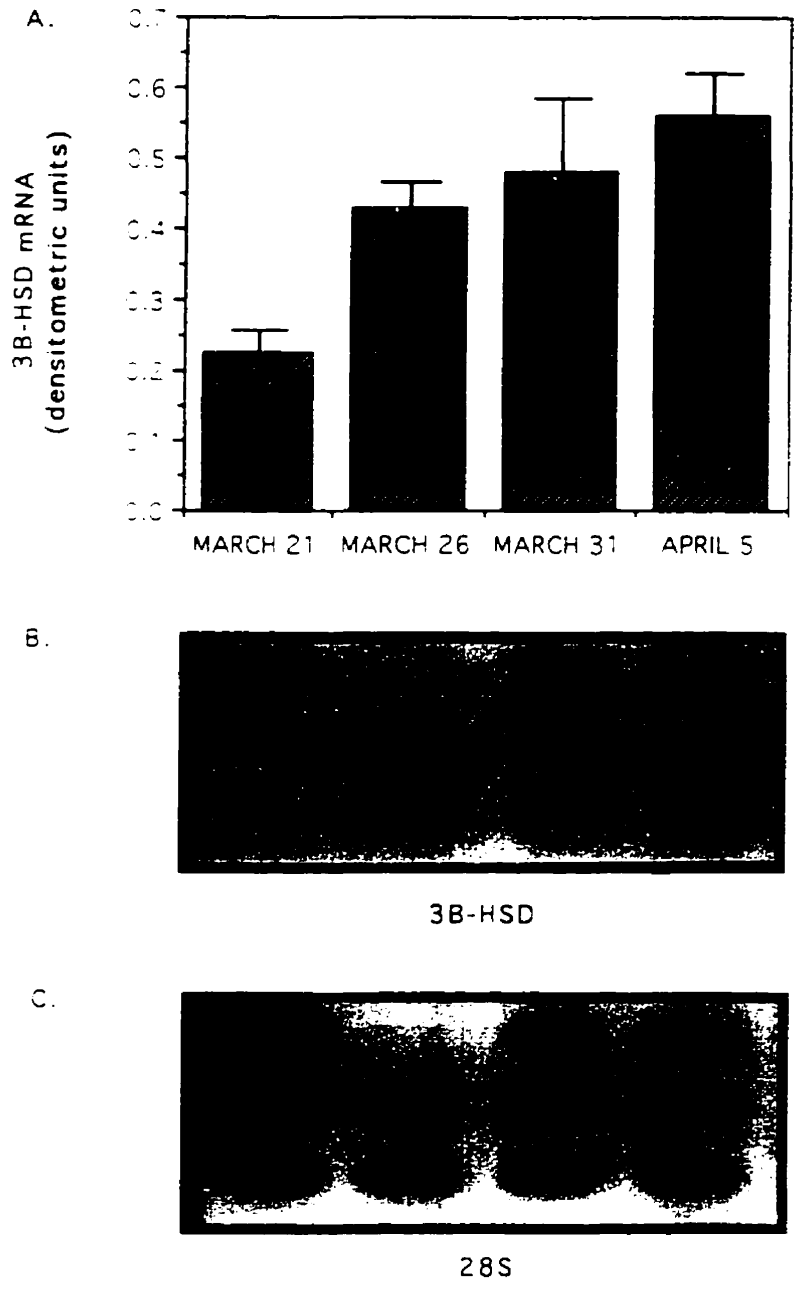
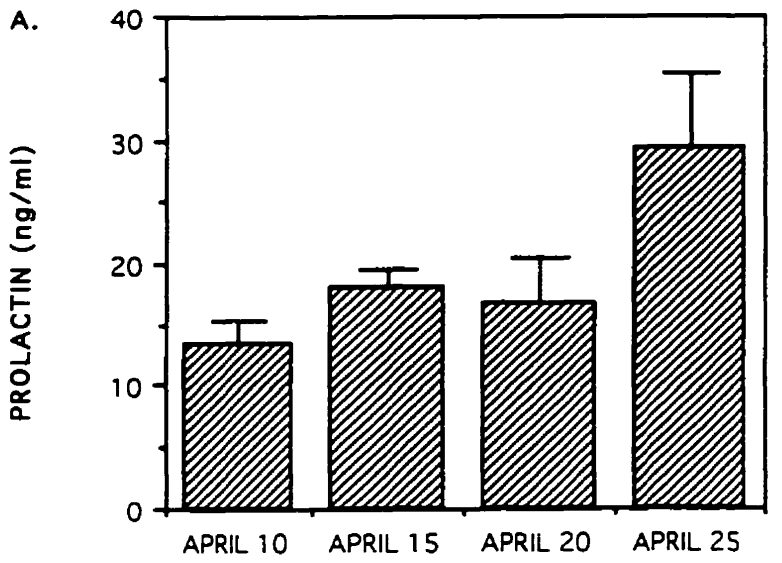

A.
3B-HSD mRNA
(densitometric units)
0.6
MARCH 21
MARCH 26
MARCH 31
APRIL 5
B.
3B-HSD
C.
28S

Figure 5. Mean (+ SEM) of serum (a). prolactin, and (b). progesterone concentrations in pregnant mink during the post-implantation stage (April 10 to April 15) of gestation. Prolactin and progesterone levels were determined by double antibody RIA.


A.
PROLACTIN (ng/ml)
40
30
20
10
0
APRIL 10
APRIL 15
APRIL 20
APRIL 25

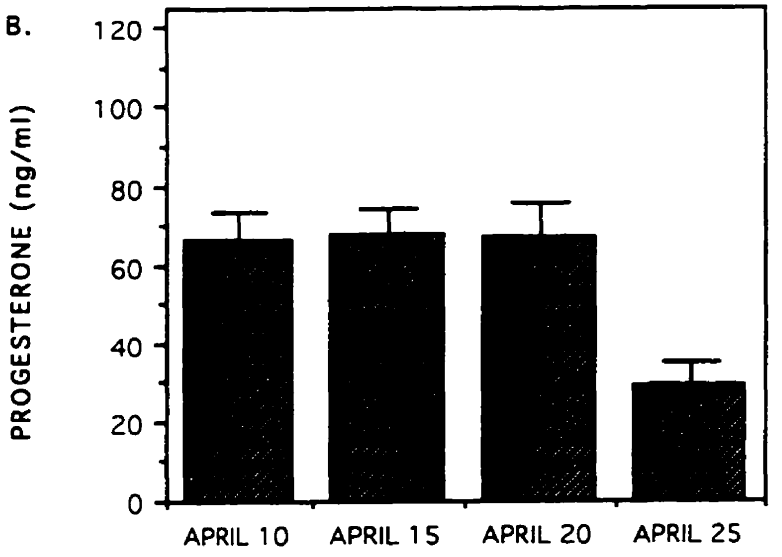
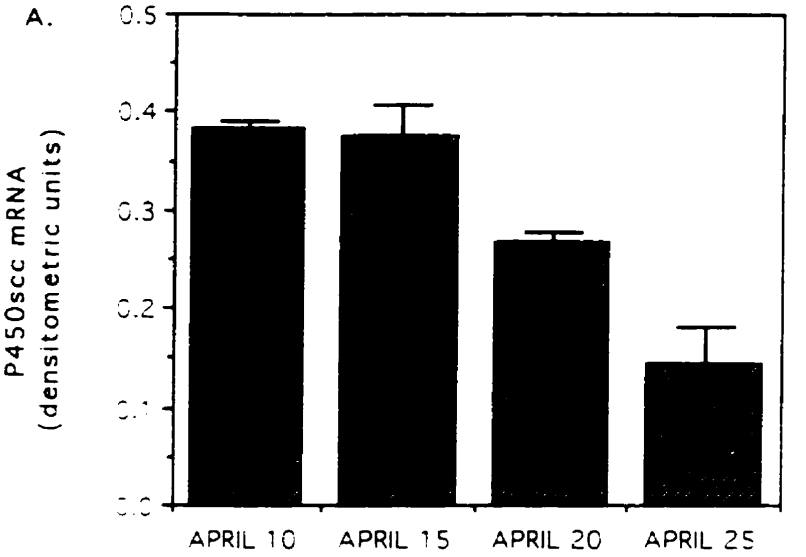
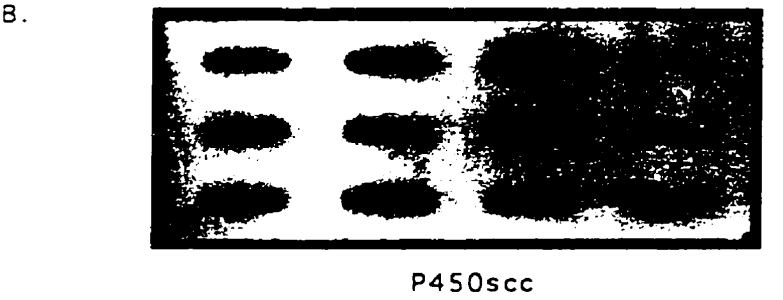

B.
PROGESTERONE (ng/ml)
120
100
80
60
40
20
0
APRIL 10
APRIL 15
APRIL 20
APRIL 25

Figure 6. (a). Abundance of P450scc mRNA (mean + SEM) in pregnant mink CL during the post-implantation stage (April 10 to April 25) of gestation. Abundance of mRNA for P450scc was determined by slot blot analysis using 28S as a constitutive control. P450scc mRNA levels are expressed in arbitrary densitometric units. (b). Autoradiograph of P450scc labeled slot blot. (c). Autoradiograph of human ribosomal 28S labeled slot blot.


A.
0.5
0.4
0.3
0.2
P450scc mRNA
(densitometric units)
APRIL 10
APRIL 15
APRIL 20
APRIL 25


B.
P450scc

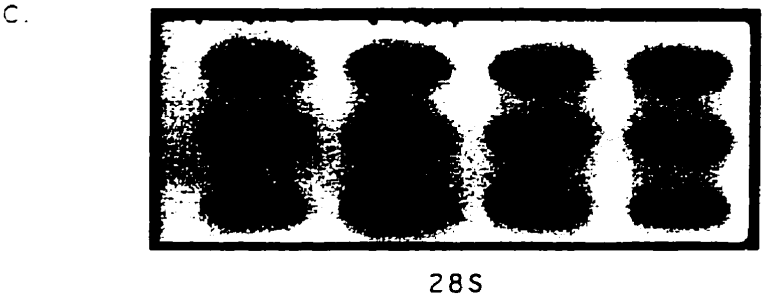
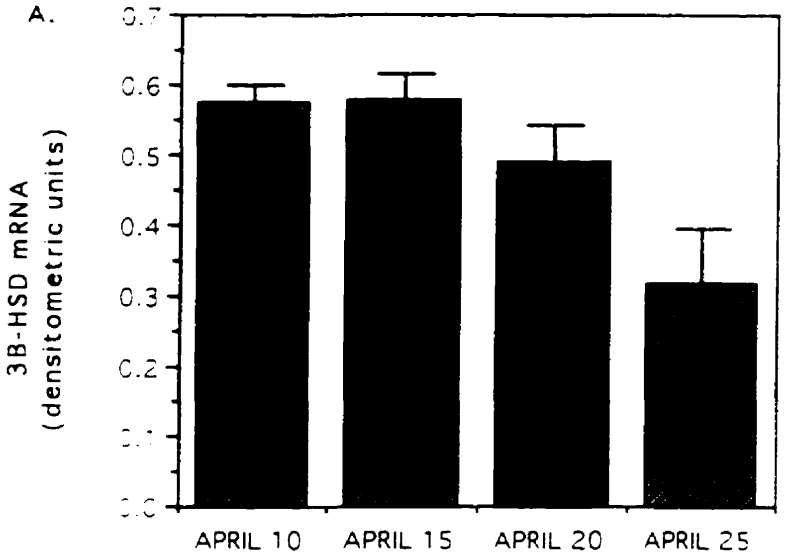

C.
28S

Figure 7. (a). Abundance of 3β-HSD mRNA (mean + SEM) in pregnant mink CL during the post-implantation stage (April 10 to April 25) of gestation. Abundance of mRNA for 3β-HSD was determined by slot blot analysis using 28S as a constitutive control. 3β-HSD mRNA levels are expressed in arbitrary densitometric units. (b). Autoradiograph of 3β-HSD labeled slot blot. (c). Autoradiograph of human ribosomal 28S labeled slot blot.


A.
3B-HSD mRNA
(densitometric units)
0.7
0.6
0.5
0.4
0.3
0.2
APRIL 10
APRIL 15
APRIL 20
APRIL 25


B.
3B-HSD


C.
28S

Figure 8. Mean (+ SEM) serum (a). prolactin and (b). progesterone concentrations in pregnant mink during embryonic diapause, CL reactivation and early post-implantation gestation, determined by RIA. Solid boxes represent animals implanted with Alzet osmotic minipumps which released 2 mg/day bromocryptine. Empty boxes represent control animals implanted with minipumps containing saline.

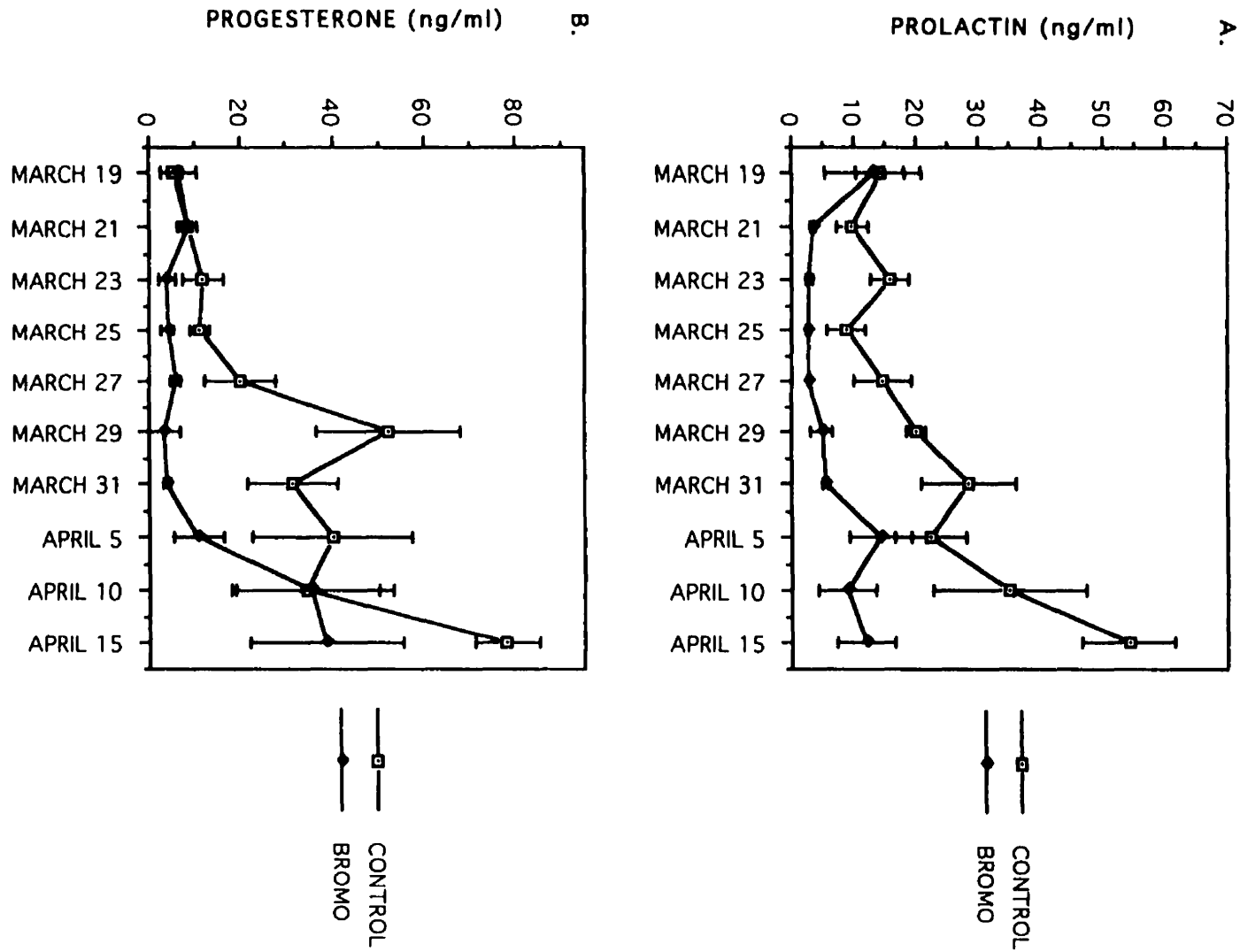
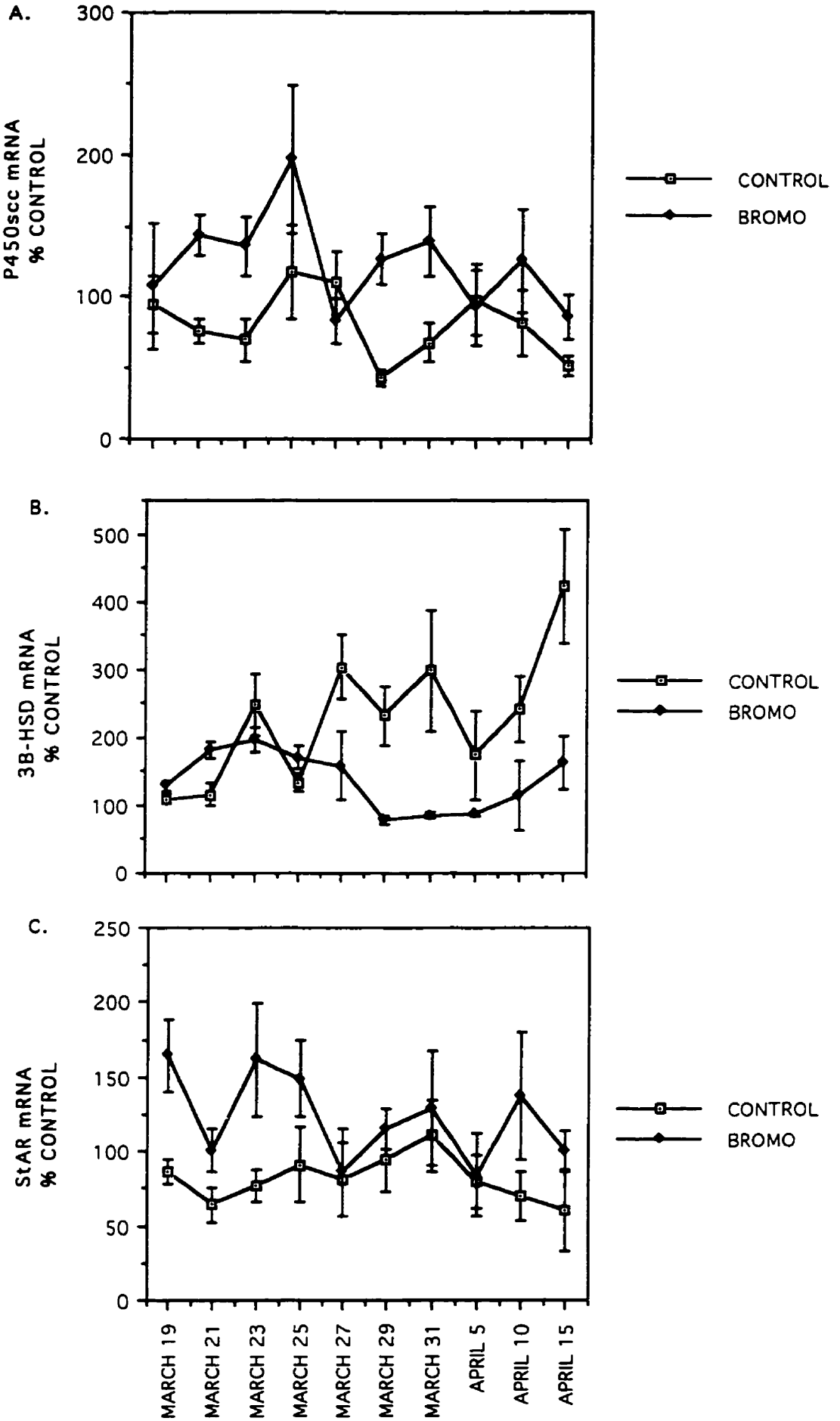

PROLACTIN (ng/ml)
A.
0
10
20
30
40
50
60
70
MARCH 19
MARCH 21
MARCH 23
MARCH 25
MARCH 27
MARCH 29
MARCH 31
APRIL 5
APRIL 10
APRIL 15
CONTROL
BROMO
PROGESTERONE (ng/ml)
B.
0
20
40
60
80
MARCH 19
MARCH 21
MARCH 23
MARCH 25
MARCH 27
MARCH 29
MARCH 31
APRIL 5
APRIL 10
APRIL 15
CONTROL
BROMO

Figure 9. Abundance of ovarian (a). P450scc mRNA, (b). 3β-HSD mRNA, (c). StAR mRNA (mean + SEM) in pregnant mink during embryonic diapause and CL reactivation. Abundance of mRNA for P450scc, 3β-HSD and StAR were determined by slot blot or Northern analysis using 28S as a constitutive control. Messenger RNA levels are expressed as a percentage of a control derived from a pool of ovaries taken from anestrous animals.


A.
P450scc mRNA
% CONTROL
300
200
100
0
CONTROL
BROMO
B.
3B-HSD mRNA
% CONTROL
500
400
300
200
100
0
CONTROL
BROMO
C.
StAR mRNA
% CONTROL
250
200
150
100
50
0
CONTROL
BROMO
MARCH 19
MARCH 21
MARCH 23
MARCH 25
MARCH 27
MARCH 29
MARCH 31
APRIL 5
APRIL 10
APRIL 15

4.6 DISCUSSION

We originally hypothesized that failure of the delay mink CL to produce progesterone was due to a lack of the enzyme P450scc for the conversion of cholesterol to pregnenolone, an important rate limiting step in steroidogenesis. However, in spite of dramatic changes in progesterone levels over the course of mink gestation, there were no changes in the abundance of luteal mRNA for P450scc during the stage of mink gestation associated with CL reactivation. Thus the results from this study support the view that mRNA for P450scc is constituitively expressed in the mink corpus luteum during embryonic diapause, CL reactivation and early post-implantation gestation. This agrees with observations in a number of species, including the rat (Goldring *et al.*, 1987; Richards *et al.*, 1987; Hickey *et al.*, 1988) and the macaque (Basset *et al.*, 1991), in which the rise and fall in progesterone production as a result of growth and regression of the CL was not reflected in remarkable changes in the amount of hybridizable transcript for P450scc. However, the constitutive expression of P450scc is not universal, since changes in the abundance of P450scc through the lifespan of the CL have been reported in the cow (Rodgers *et al.*, 1986) and sheep (Belfiore *et al.*, 1994; Juengel *et al.*, 1994).

We further hypothesized that the luteotropic effects of prolactin may include alterations in the abundance of P450scc gene products. Again, the current study reveals that neither the increase in endogenous prolactin associated with the vernal equinox nor the suppression of prolactin by bromocryptine treatment altered P450scc mRNA levels from those seen in delay CL. It remains possible that there could have been translational differences or changes in enzyme activity in the absence of variation in P450scc mRNA. However, Hinshelwood *et al.* (1993) reported that the major control of the production of P450scc is largely transcriptional, as changes in amounts of hybridizable transcript mirror the shifts in the quantity of P450scc protein and activity throughout the ovarian cycle.

In contrast to P450scc, the abundance of 3β-HSD mRNA changed dramatically over the course of mink gestation and was positively correlated with progesterone levels. Levels increased 2.5 fold in association with luteal reactivation and as circulating progesterone levels fell during late gestation, 3β-HSD mRNA declined concurrently. There have been reports that the expression of 3β-HSD mRNA in the ovary is constitutive (Readhead *et al.*, 1983; Gore-Langton *et al.*, 1988). However, Couet *et al.* (1990 and 1991) demonstrated that in the bovine ovary, 3β-HSD is strongly modulated during the estrous cycle and that progesterone production by the CL appears to be regulated to a large extent by changes in 3β-HSD gene transcription and/or 3β-HSD mRNA stability. In addition, 3β-HSD mRNA levels per CL have also been shown to be positively correlated to serum concentrations of progesterone in the sheep (Juengel *et al.*, 1994).

In the mink, prolactin is essential for the termination of embryonic diapause, for the reactivation of the corpus luteum and for the maintenance of CL function throughout gestation (Murphy *et al.*, 1980 and 1981). The results from the current experiment suggest that the abundance of 3β-HSD mRNA in the mink is under the influence of serum prolactin levels. During CL reactivation, the 2 fold increase in prolactin levels coincided with the increase in 3β-HSD mRNA levels. More importantly, suppression of endogenous prolactin by bromocryptine, completely prevented the normal pre-implantation rise in 3β-HSD mRNA levels. The failure of ovaries in bromocryptine treated animals to produce 3β-HSD mRNA does not appear to be due to the involution or the demise of the CL, since no concomitant effects on either P450scc or StAR mRNA levels were seen. Hence, among the steroidogenic elements evaluated, the luteotropic effects of prolactin appeared to be specific to 3β-HSD mRNA in this species.

An incongruity arises during late post-implantation gestation (after April 15), at which time prolactin levels continue to rise while 3β-HSD mRNA levels fall. This indicates that the effects of prolactin on 3β-HSD may vary not only between the species and tissue studied, but also with respect to the dose employed. This hypothesis is in agreement with findings in human granulosa cells by Tremblay *et al.* (1991). Tremblay *et al.* (1991) reported that prolactin induced a dose dependent decrease in both progesterone and 3β-HSD mRNA. Dombrowicz *et al.* (1992) found a prolactin regulated dose-dependent increase in the number of immunopositive cells for 3β-HSD in the hypophysectomized rat testes. Treatment of hypophysectomized female rats with 1 mg of ovine prolactin twice daily exerted a potent inhibitory effect on ovarian 3β-HSD mRNA levels (Martel *et al.*, 1990; Labrie *et al.*, 1991 and 1992). Co-treatment of these animals with human chorionic gonadotropin (hCG) resulted in a partial reversal of these inhibitory effects (Martel *et al.*, 1990; Labrie *et al.*, 1991 and 1992). The potent inhibitory effects of prolactin on 3β-HSD mRNA in the rat ovary are surprising since prolactin is essential to the control and maintenance of the rat CL during the first 6 days of pregnancy (Morishige and Rothchild, 1974). This discrepancy, however, may be due to the large dose of prolactin used in the hypophysectomized rat experiments (1 mg of prolactin 2 x daily).

Although the present study was not designed to determine the effects of variant levels of prolactin on ovarian function, it is interesting to speculate that low doses may be stimulatory to the mink CL, while high levels may be inhibitory. Intact mink implanted with minipumps which continually released ovine prolactin, displayed early reactivation of the mink CL as indicated by the precocious increases in progesterone secretion (Murphy *et al.*, 1990). However, continued prolactin treatment induced a premature decline in progesterone levels relative to untreated controls. Results from the current experiment showed that not only did luteal 3β-HSD mRNA levels decline during the later part of

gestation in association with elevated levels of prolactin but P450scc mRNA levels fell as well. If high levels of prolactin are in fact responsible for the decline in 3β-HSD and P450scc mRNA seen here during late post-implantation gestation, their change may reflect the overall demise of the CL rather than a specific change in the regulation of these genes.

The final objective of this study was to monitor changes in the abundance of StAR mRNA in the mink ovary during CL reactivation and early post-implantation gestation. StAR is an acutely regulated, cycloheximide-sensitive, mitochondrial protein which is believed to be involved in the transport of cholesterol across the mitochondrial membranes to the site of P450scc enzyme activity (Clark *et al.*, 1994; Clark *et al.*, 1995; Lin *et al.*, 1995). Since StAR is a regulated protein necessary for the provision of substrate in the P450scc enzyme system we hypothesized that alterations in its abundance may play a role in CL reactivation in the mink. However, the results of the current experiment showed that the abundance of StAR mRNA did not change over the course of CL reactivation or early post-implantation gestation. In addition, alterations of endogenous prolactin levels had no effect on the abundance of StAR mRNA. It appears that StAR is regulated by mechanisms other than prolactin in the mink ovary and does not vary in association with CL reactivation.

In conclusion, the termination of embryonic diapause, CL reactivation and the pre-implantation stimulation of progesterone secretion in the mink ovary do not involve transcriptional changes and/or changes in RNA stability of either P450scc or StAR mRNA. In addition, alterations of endogenous prolactin levels have no effect on the abundance of mRNA for either of these genes. In contrast, 3β-HSD appears to be tightly regulated in the mink ovary and is positively correlated to ovarian progesterone production. Alterations in endogenous prolactin levels had significant effects on 3β-HSD mRNA abundance either through an increase in gene transcription and/or changes in mRNA stability. We concluded that one possible effect of prolactin on the mink CL is to stimulate progesterone production by increasing the amount of 3β-HSD enzyme available for conversion of pregnenolone to progesterone.

4.7 ACKNOWLEDGMENTS

We would like to thank Drs. T. Wise, F. Labrie and D. Stocco for the provision of the cDNA probes utilized in these experiments. We would also like to thank Mr. M. Morrow for animal care. Technical assistance was provided by Mrs. M. Dobias Goff. This study was supported by NSERC and MRC grants to BDM.

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4.9 STATEMENT OF PARTICIPATION

Experimental Design - Deborah Douglas, Bruce Murphy

Animal Handling - Deborah Douglas, Jianhua Song

Sample Collection - Deborah Douglas, Jianhua Song

Northern and Slot Blot Analyses - Deborah Douglas

Progesterone Assay - Jianhua Song

Prolactin Assay - Pat Concannon

Statistical Analysis - Deborah Douglas

Interpretation of Results - Deborah Douglas, Bruce Murphy

Paper - Deborah Douglas

4.10 CONNECTOR

In Papers # 1 and 2, a pregnant whole animal model was used to determine changes in the abundance of mRNA for luteotropic hormone receptors and a number of steroidogenic elements during embryonic diapause, CL reactivation and early post-implantation gestation. The animals in these experiments were exposed to endogenous prolactin levels or had their prolactin levels suppressed by treatment with the dopamine agonist bromocryptine. The results of Paper #1 revealed that PRLr and LHr mRNA levels varied during embryonic diapause, CL reactivation and early post-implantation gestation and that these levels could be effected by the suppression of endogenous prolactin levels. The results of Paper #2 revealed that changes in the abundance of P450scc and StAR mRNA do not appear to be important for the reactivation of the mink CL since the suppression of endogenous prolactin levels did not effect their abundance. Further, the results revealed that 3β-HSD mRNA levels did vary during CL reactivation and early post-implantation gestation and that the pre-implantation rise in 3β-HSD mRNA could be prevented by treatment with bromocryptine.

The principal objective in Paper #3 was to further study the effects of prolactin on the luteotropic hormone receptors and steroidogenic elements using an anestrous mink model. In this model anestrous animals were treated with eCG and GnRH to induce follicular development and ovulation. The animals were then treated with prolactin or left as untreated controls. This model allowed us to study the effects of prolactin alone on the mink ovary, while eliminating other factors from the pituitary or uterus which may also be having effects on the mink ovary.

INDUCTION OF FOLLICULAR DEVELOPMENT, OVULATION AND CORPUS LUTEUM FUNCTION IN THE ANESTROUS MINK (*Mustela vison*).

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Key Words: Mink, anestrous, eCG, prolactin, P450scc, 3β-HSD, StAR, PRLr, LHr.

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5.2 ABSTRACT

Follicular development, ovulation and corpus luteum (CL) function were induced in anestrous mink, five months prior to their normal breeding season. Nulliparous female mink were given a 100 IU injection of equine chorionic gonadotropin (eCG) to induce follicular development (Day 0). Three days later they were given an ovulatory dose of gonadotropin releasing hormone (10 μg/animal, GnRH) plus anti-eCG antibodies (125 IU) to neutralize the residual effects of the original eCG injection (Day 3). The mink were then divided into two treatment groups. The first group received 25 IU of ovine prolactin/day beginning on Day 5 and continuing for up to 8 days (Day 12). The second group received no further treatment and acted as a control. The ovaries and a terminal blood sample were collected from three animals per group on each day of the experiment. Total ribonucleic acid (RNA) was purified from whole ovaries and used for Northern and slot blot analysis to determine the abundance of mRNA for P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), steroidogenic acute regulatory protein (StAR) and the luteotropic hormone receptors for prolactin (PRLr) and luteinizing hormone (LHr). In addition, serum progesterone levels were determined by double antibody radioimmunoassay (RIA). The abundance of mRNA for P450scc did not vary in the non-prolactin treated group over the experimental period. However, P450scc levels declined in the prolactin treated group by 60-70% and were significantly ($P<0.05$) lower than the non-prolactin treated control. The abundance of 3β-HSD mRNA increased after the eCG injection but decreased after the ovulatory dose of GnRH. Treatment with prolactin caused a transient 3 fold increase in the abundance of 3β-HSD mRNA levels over that of the non-treated prolactin controls. After two days of prolactin treatment the abundance of LHr mRNA increased 3 fold. The prolactin treated group had significantly ($P<0.05$) higher levels of LHr mRNA than the non-prolactin treated group. The levels of LHr mRNA did not change in the control group. Although follicular development, ovulation and changes in corpus luteum function occurred in response to exogenous hormone treatment, progesterone did not vary over the course of the experimental period or in response to prolactin treatment. In conclusion, it was possible to induce follicular development and ovulation in anestrous mink using the above protocol. Treatment of these mink with prolactin increased ovarian mRNA levels of 3β-HSD and LHr, had no effect on StAR mRNA and reduced the abundance of P450scc. Although the enzymes necessary for progesterone production were present in the ovaries and presumably the induced CL, prolactin alone was unable to stimulate progesterone production in these animals.

5.3 INTRODUCTION

The mink is a semi-domesticated carnivore which retains a strict pattern of reproductive seasonality. The breeding season begins at the end of February, in the Northern hemisphere, and continues for most of the month of March (Hansson, 1947; Enders, 1952; Pilbeam *et al.*, 1979). It is characterized by induced ovulation, superfetation and a period of embryonic diapause also known as delayed implantation (Hansson, 1947; Enders, 1952, Shackleford, 1952). One of the unique characteristics of the mink is it can mate and ovulate more than once during the breeding season (Hansson, 1947; Enders, 1952). Ovulation occurs 36-48 hours after the mating stimulus (Hansson, 1947, Enders, 1952). If fertilization occurs, the resulting blastocysts develop to the 200-400 cell stage, migrate to the uterus and enter diapause (Mead, 1981). Seven to ten days later the mink can remate, ovulate again and produce a second set of viable embryos (Hansson, 1947). During embryonic diapause the CL is inactive and produces little or no progesterone, it is only after the vernal equinox that the CL is reactivated and begins to produce progesterone (Moller, 1973). Several studies have shown that prolactin is the principal luteotropic hormone in the mink and is responsible for the reactivation of the pre-implantation mink CL. It is also necessary for its maintenance and function after implantation (Papke *et al.*, 1981; Martinet *et al.*, 1981; Murphy *et al.*, 1980, Murphy *et al.*, 1981).

In order to further studies on the molecular effects of prolactin on the mink corpus luteum, a reliable method for the induction of estrus and ovulation outside of the normal breeding season was needed. Equine chorionic gonadotropin, also known as pregnant mare serum gonadotropin (PMSG), has been used as an agent for the stimulation of folliculogenesis in a number of carnivores including the cat (Goodrowe *et al.*, 1987; Donoghue *et al.*, 1992), dog (Arnold *et al.*, 1989), fox (Douglas *et al.*, 1993) and mink (Murphy *et al.*, 1987; Wehrenberg *et al.*, 1989; Stagg *et al.*, 1992). Equine chorionic gonadotropin is a glycoprotein hormone that has the activity of both follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in species other than the horse (reviewed by Murphy and Martinuk, 1991). Relative to other gonadotropins eCG persists in the blood for relatively long periods, primarily due to the large sialic acid content of glycosylated tail of the β-chain subunit (Murphy and Martinuk, 1991; Martinuk *et al.*, 1991). The persistence of eCG provides the benefit of only having to use a single injection of the hormone, although the long half life may result in the overstimulation and the subsequent reduction in the ovulatory rates of treated animals (Monniaux *et al.*, 1984). In other species the administration of mono- or polyclonal antibodies against eCG have been used to neutralize the effects of eCG and improve reproductive success (Dhondt *et al.*, 1978; Zeitoun *et al.*, 1991, Douglas *et al.*, 1993).

The objectives of this study were to 1) develop a protocol for the induction of folliculogenesis and ovulation in the mink outside of the normal breeding season and to 2) determine the effects of exogenous prolactin on the abundance of mRNA for the steroidogenic enzymes P450scc and 3β-HSD, the steroidogenic regulatory protein StAR and cell surface receptors for the hormones LH and PRL.

5.4 MATERIALS AND METHODS

5.4.1 PRELIMINARY EXPERIMENT

Adult anestrous mink were injected with 100 IU of eCG (Stimukron: Sanofi, PQ) on the first day of the experiment. Three days later the same animals received an ovulatory dose of GnRH (Factrel: Ayerst). Eight days after ovulation, the animals were sacrificed and the ovaries collected. The ovaries were placed in ice cold saline and the CL isolated and counted under a dissecting microscope.

5.4.2 EXPERIMENTAL DESIGN

Adult female mink were maintained on a commercial mink ranch (Morrow Fourrures; St Paul d'Abbotsford, PQ). They received a standard wet mink ration and water *ad libitum*. In November, follicular development was induced in anestrous females by treatment with 100 IU of eCG (Stimukron: Day 0). Three days later an ovulatory dose of 10 μg/animal of GnRH (Factrel) was given. In addition, 125 IU of anti-eCG antibodies (Neutra-PMSG: Intervet) were given on Day 3 to neutralize the residual effects of the original eCG injection. The animals were then divided into 2 groups. The first group received no further treatment and acted as a control. Beginning on Day 5 until Day 12, the second group of animals were given daily injections of 25 IU ovine prolactin (Sigma, St Louis, MO). The ovaries and a terminal blood sample were collected from 3 animals per group on each day of the experiment. Macroscopic observation of the ovaries confirmed the presence of ovulation sites and CL on the ovaries. The ovaries were then placed in 4 M guanidinium isothiocyanate (GITC: Gibco/BRL, Burlington, ON) plus 0.12 M 2-mercaptoethanol (Sigma) and snap frozen in liquid nitrogen and stored at -70 C until RNA purification.

5.4.3 NORTHERN AND SLOT BLOT ANALYSES

Total RNA was purified by cesium chloride (CsCl) gradient ultracentrifugation as described by Chirgwin *et al.* (1979). Tissue samples were thawed and homogenized with a PT 3000 polytron (Brinkmann, Rexdale, ON). The homogenate was then layered onto a 5.7 M CsCl (Sigma) gradient and centrifuged at 32,000 rpm (174,000g) using a SW-41 rotor (Beckman, Mississauga, ON), for 20 hours at 22 C. The RNA pellet was dissolved and precipitated twice in 0.1 volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol. After the final washing the pellet was dissolved in diethyl pyrocarbonate (DEPC: Sigma)-treated distilled water and stored at -70 C. The RNA concentration for each sample was determined by spectrophotometric measurement at an absorbance of 260 nm.

For Northern analysis, 15 μg of total RNA was size fractionated on a 1% agarose denaturing formaldehyde gel and stained with ethidium bromide (Sigma; Sambrook *et al.*, 1989). The RNA was transferred by capillary action overnight using 20 x SSC (1 x SSC = 150 mM NaCl, 15 mM trisodium citrate pH 7.0) onto a nylon membrane (Hybond-N: Amersham, Oakville, ON). The RNA was cross-linked to the membrane using GS gene linker UV chamber (BioRad, Mississauga, ON).

Slot blot analysis used 6 μg of total RNA per sample. The total sample volume was made up to 50 μl with DEPC-treated water. 150 μl of denaturing solution (98 μl deionized formamide, 32 μl 37% formaldehyde and 20 μl of 10 x MOPS (0.2 M 3-[N-morpholino] propanesulphonic acid, 0.5 M sodium acetate pH 7.0, 0.01 M sodium ethylenediaminetetra acetic acid (EDTA)) was added to the samples and heated at 65 C for 10 minutes. An equal volume of ice-cold 20 x SCC was added to the samples, which were applied to a nylon membrane (Hybond-N: Amersham) using a slot blot manifold (BioRad).

The hybridization procedure consisted of rinsing the membranes in 5 x SSPE (1 x SSPE = 180 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) then prehybridizing for 30 minutes at 60-65 C in hybridizing solution (5 x SSPE, 5 x Denhardt's solution: 0.5 % sodium dodecyl sulfate (SDS), 10% dextran sulfate). Denatured herring sperm DNA (Sigma) at a concentration of 10 μg/ml was then added to the hybridizing solution and the prehybridization period continued for a minimum of 3 more hours. Complementary DNA probes (porcine P450scc: Mulheron *et al.*, 1989; human: 3β-HSD: Luu-The *et al.*, 1989; mouse StAR: Clark *et al.*, 1994; mink PRLr: Douglas *et al.*, 1996b; mink LHr: Douglas *et al.*, 1996b) were labeled by random primer extension (Boeringer Mannheim, Laval, PQ) with [^{32}P]-dCTP (Dupont, Mississauga, ON). Once labeled, the probes were denatured and added to the hybridization solution. The incubation was continued overnight at 60-65 C. Membranes were washed twice, 15 minutes per wash, with 2 x SSPE plus 0.1% SDS at room temperature and twice at 60 C. The membranes

were sealed in hybridizing bags with a small amount of 5 x SSPE and exposed cassettes to Kodak XAR-5 film (Rochester, NY) with intensifying screens. All membranes were rehybridized with a ribosomal 28S probe (Gonzales *et al.*, 1985). Autoradiographic images were digitized (FotoDyne) and analyzed using Collage software. Results are expressed as a ratio between the cDNA probe of interest and ribosomal 28S. These arbitrary units were then expressed as a percentage of a pool of RNA from unstimulated ovaries collected from anestrous animals, which was present on all the slot blots.

5.4.4 PROGESTERONE ASSAY

Mink serum progesterone concentrations were determined by liquid-phase radioimmunoassay after extraction in 10 volumes of hexane (BDH, Darmstadt, WG). Extraction recoveries ranged between 95-98%. An antiserum raised against 4-pregnen-11α-o-13,20dione hemisuccinate bovine serum albumin, kindly provided by Dr. A Goff (Lafrance and Goff, 1985) was used as the first antibody. Progesterone-11α-glucuronide-[^{125}I] iodotryramine (Amersham, Oakville, ON) was used as tracer and goat anti-rabbit IgG (Prince Laboratories, Toronto, ON) as second antibody. The sensitivity of the assay was determined to be 6 pg. The intra- and inter-assay coefficients of variation were 12.2 and 12.3%, respectively.

5.5 RESULTS

Results of the preliminary experiment revealed that the hormone treatment regime utilized in this study resulted in follicular development and ovulation in anestrous mink. The number of CL isolated from mink ovaries treated with eCG and GnRH varied among and between the two ovaries. The number of CL isolated ranged between 0 and 21, with the average being 11 CL/ovary.

Since the hormone regime used in the preliminary experiment resulted in follicular development and ovulation in anestrous mink, it was used in subsequent studies to determine the effects of prolactin on the mink ovary. To limit the super stimulatory effects of eCG , which has a half life in the order of days (Martinuk *et al.*, 1991), on ovarian function, an anti-eCG antibody was also given.

Initially this model was used to study the effects of prolactin on the abundance of mRNA for a variety of steroidogenic elements, including P450scc, 3β-HSD and StAR. The abundance of mRNA for P450scc did not vary in the non-prolactin treated group over the experimental period. However, P450scc levels declined in the prolactin treated group by 60-70% and were significantly ($P<0.05$) lower than the non-prolactin treated control (Figure 1). The abundance of 3β-HSD mRNA increased after the eCG injection but

decreased after the ovulatory dose of GnRH. Treatment with prolactin caused a transient 3 fold increase in the abundance of 3β-HSD mRNA levels over that of the non-prolactin treated controls between Days 8 to 10 (Figure 2). There were no differences in the abundance of mRNA for the cholesterol transport protein StAR over the course of the experimental period. In addition, there was no difference in the abundance of StAR mRNA between the prolactin treated and control groups (Figure 3).

The experimental model was also used to determine the effects of prolactin on the luteotropic hormone receptors for LH and prolactin. After two days of prolactin treatment the abundance of LHr mRNA increased 3 fold and remained high through out the experimental period, while the level of LHr mRNA did not vary significantly in the control group. In addition, it was shown that the prolactin treated group had significantly ($P<0.05$) higher levels of LHr mRNA than the non-prolactin treated group (Figure 4). Prolactin treatment also resulted in an increase in the abundance of PRLr mRNA levels over that of the pre-treated controls (Figure 5).

Although follicular development, ovulation and changes in corpus luteum function occurred in response to exogenous hormone treatment, serum progesterone levels did not vary over the course of the experimental period and did not change in response to prolactin treatment.

Figure 1. Mean (+ SEM) of P450scc mRNA abundance in ovarian tissue collected from anestrous mink treated with 100 IU eCG (Day 1), 10 μg GnRH (Day 3) and 125 IU anti-eCG (Day 3). Open squares represent control animals. Solid squares represent animals treated with 25 IU of ovine prolactin between Days 5 and 12.

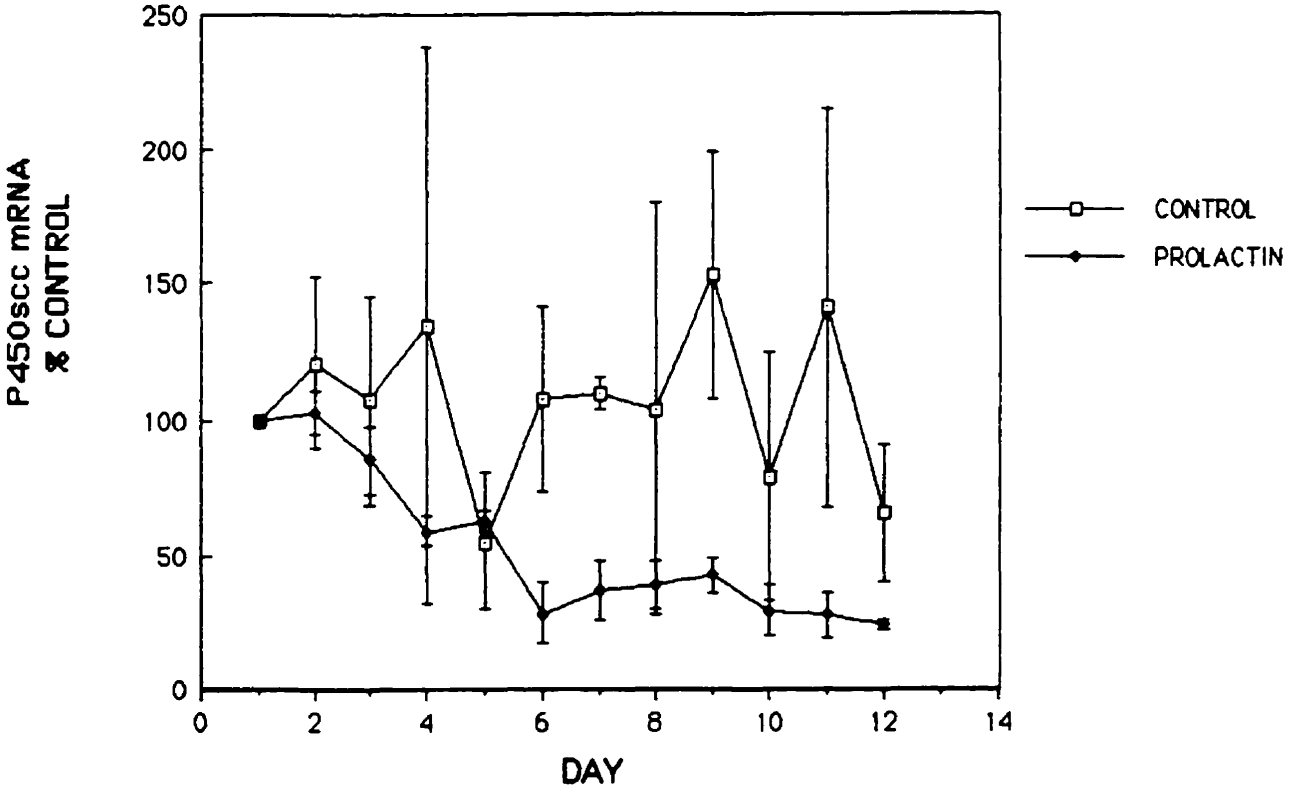

P450scc mRNA
% CONTROL
250
200
150
100
50
0
0
2
4
6
8
10
12
14
DAY
CONTROL
PROLACTIN

Figure 2. Mean (+ SEM) of 3β-HSD mRNA abundance in ovarian tissue collected from anestrous mink treated with 100 IU eCG (Day 1), 10 μg GnRH (Day 3) and 125 IU anti-eCG (Day 3). Open squares represent control animals. Solid squares represent animals treated with 25 IU of ovine prolactin between Days 5 and 12.

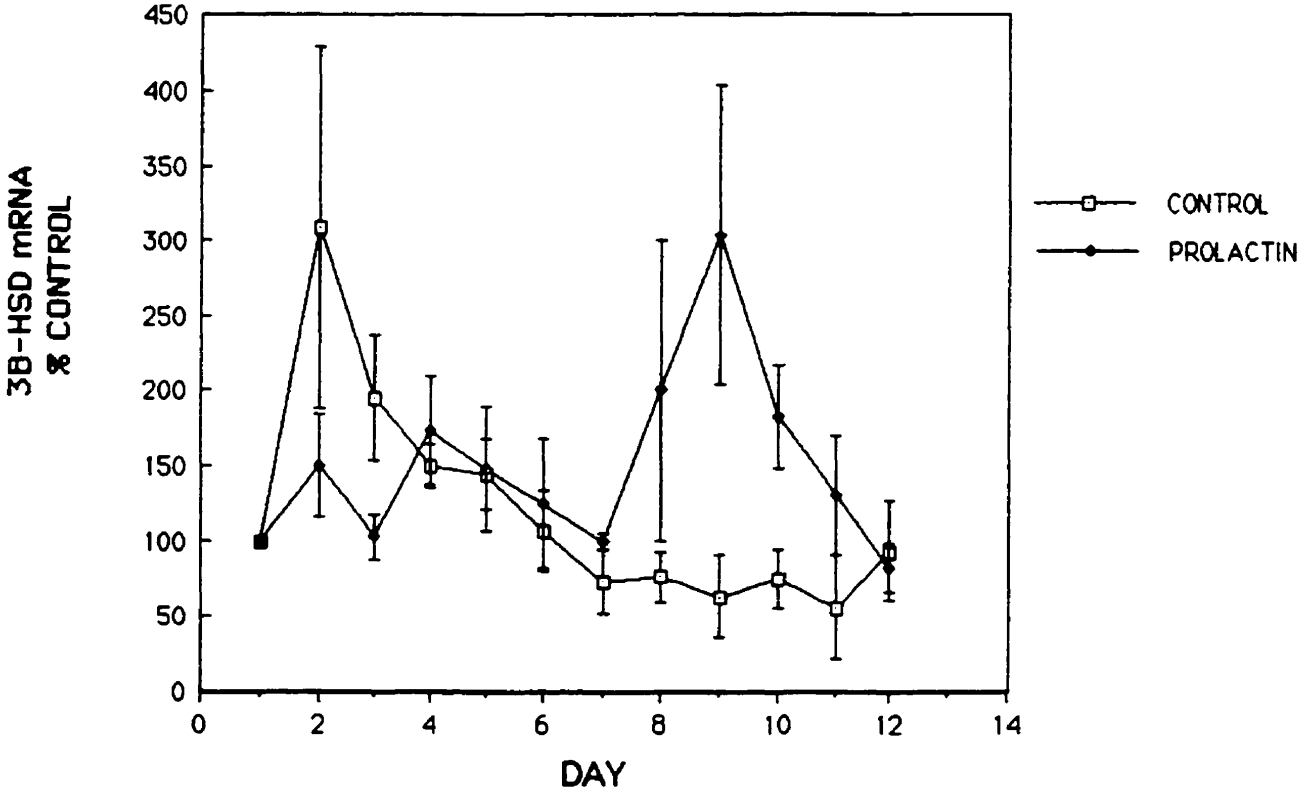

3B-HSD mRNA
% CONTROL
450
400
350
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
DAY
CONTROL
PROLACTIN

Figure 3. Mean (+ SEM) of StAR mRNA abundance in ovarian tissue collected from anestrous mink treated with 100 IU eCG (Day 1), 10 μg GnRH (Day 3) and 125 IU anti-eCG (Day 3). Open squares represent control animals. Solid squares represent animals treated with 25 IU of ovine prolactin between Days 5 and 12.

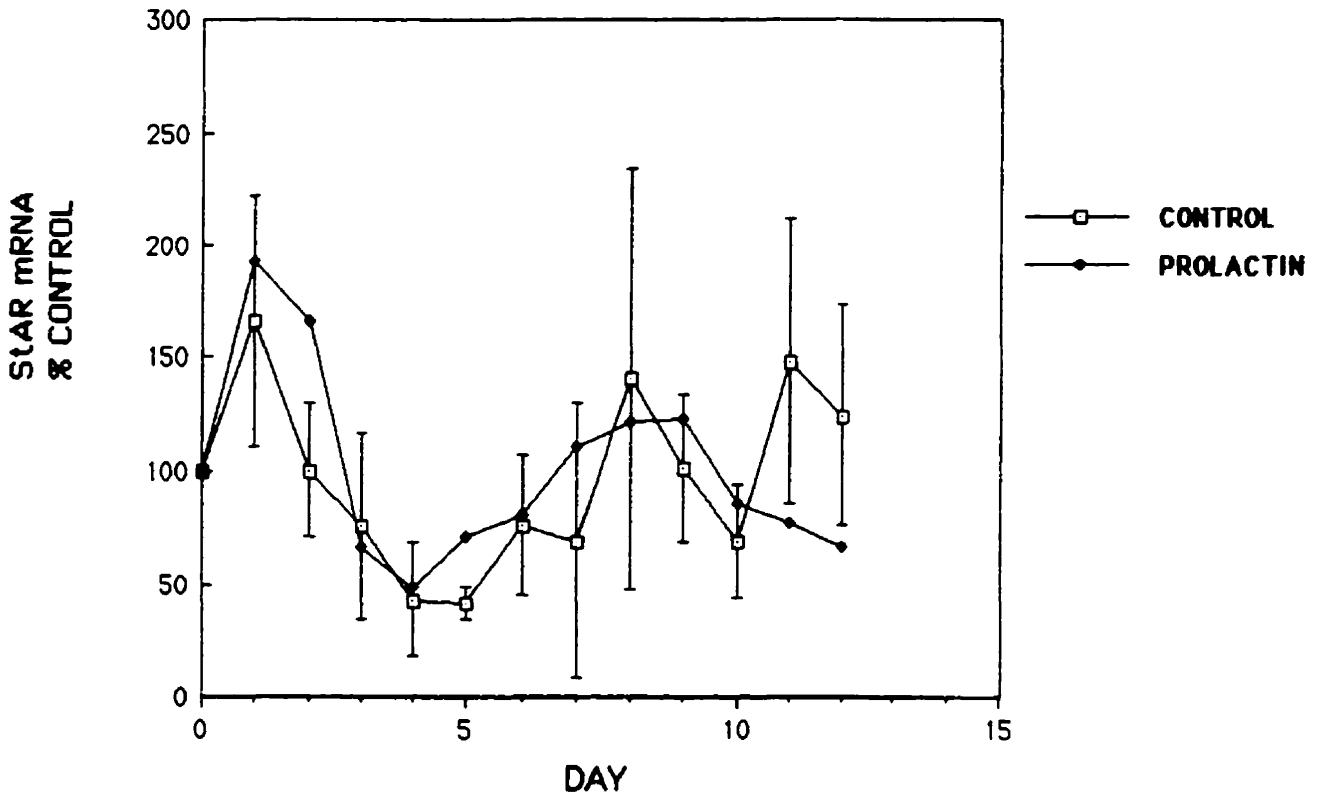

StAR mRNA
% CONTROL
300
250
200
150
100
50
0
0
5
10
15
DAY
CONTROL
PROLACTIN

Figure 4. Mean (+ SEM) of LHr mRNA abundance in ovarian tissue collected from anestrous mink treated with 100 IU eCG (Day 1), 10 μg GnRH (Day 3) and 125 IU anti-eCG (Day 3). Open squares represent control animals. Solid squares represent animals treated with 25 IU of ovine prolactin between days 5 and 12.

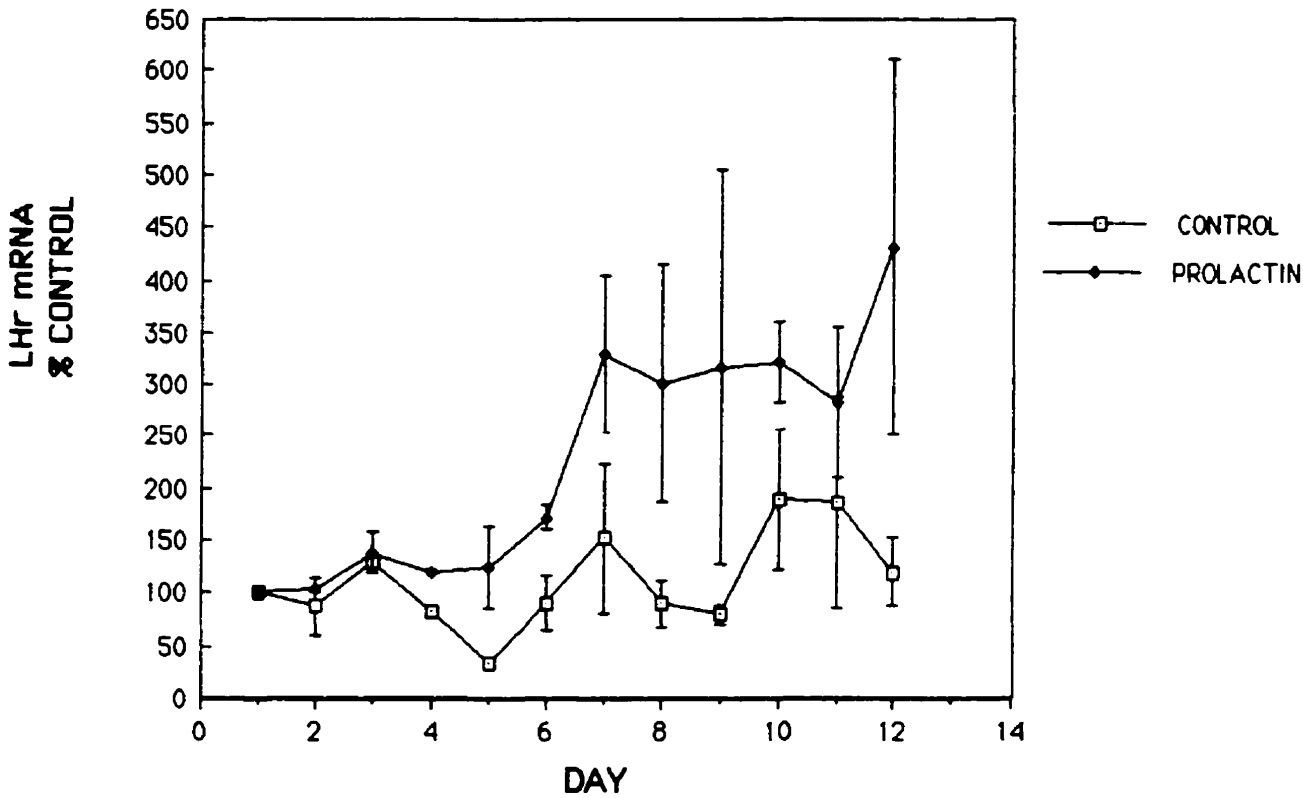
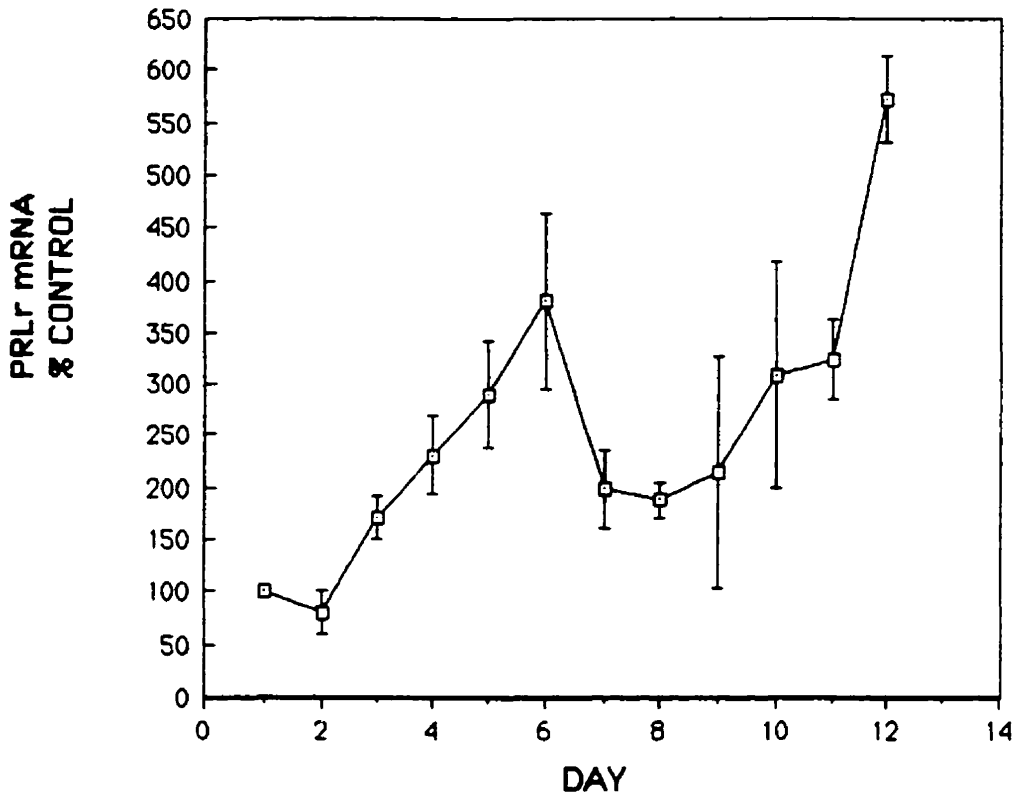

LHr mRNA
% CONTROL
650
600
550
500
450
400
350
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
DAY
CONTROL
PROLACTIN

Figure 5. Mean (+ SEM) of PRLr mRNA abundance in ovarian tissue collected from anestrous mink treated with 100 IU eCG (day 1), 10 μg GnRH (day 3), 125 IU anti-eCG (day 3) and 25 IU of ovine prolactin beginning on Day 5.


PRLr mRNA
% CONTROL
650
600
550
500
450
400
350
300
250
200
150
100
50
0
0
2
4
6
8
10
12
14
DAY

5.6 DISCUSSION

The results of the current experiment revealed that follicular development and ovulation could be induced in anestrous mink by treatment with exogenous gonadotropins. Normally during the anestrous period, follicles in the mink do not develop beyond the 0.5 mm size range (Enders, 1952), which is half of the normal preovulatory follicle size (Douglas *et al.*, 1994). Development of follicles beyond 0.5 mm appears to be critical for the development of preovulatory follicles, since little atresia occurs in follicles larger than this size and the ability of follicles to respond to the mating stimulus occurs around this stage (Douglas *et al.*, 1994). Previous studies in the mink have used eCG to stimulate follicular development in animals which have refused to mate during the normal breeding season (Murphy *et al.*, 1987; Wehrenberg *et al.*, 1989); however, in this report it was used to develop a model for studying the luteotropic effects of prolactin, without other complicating factors from the uterus and pituitary. Response to eCG treatment in the current experiment varied greatly between and within animals, with some animals ovulating up to 21 follicles, while others failed to respond at all. This variability in the response of animals to eCG treatment also has been reported for a number of other species.

Using the gonadotropin treated anestrous mink model, the effects of prolactin on various elements of the progesterone biosynthetic pathway were studied. The first element studied was cytochrome P450scc, the enzyme which, along with its electron donors adrenodoxin and adrenodoxin reductase, is responsible for the 20-hydroxylation, 22-hydroxylation and cleavage of the C20-C22 bond of cholesterol to produce pregnenolone, the principal substrate for all steroidogenesis (reviewed by Miller, 1987; Miller, 1988). The results of the current experiment revealed that prolactin treatment reduced the abundance of mRNA for this enzyme by 60-70% below the non-prolactin treated control. This result is incongruent to a previous report from our laboratory in which it was found that P450scc mRNA levels did not vary significantly during embryonic diapause, CL reactivation and early post-implantation gestation, which is a period of time during which serum prolactin levels rise dramatically in the mink (Douglas *et al.*, 1996a). In addition, treatment of pregnant mink with bromocryptine, a dopamine agonist which suppresses endogenous levels of prolactin, had no effect on P450scc mRNA levels (Douglas *et al.*, 1996a). These differences may reflect the presence of other luteotropic elements during gestation which are not present in the ovaries of animals stimulated to ovulate outside the normal breeding season. The results presented here do, however, support the finding that P450scc mRNA levels fall during late post-implantation gestation which is a time associated with the continuing rise in serum prolactin levels in the mink (Douglas *et al.*, 1996a).

The second steroidogenic element studied was 3β-HSD, which is the enzyme responsible for the oxidation and isomerization of Δ^5-3β-hydroxysteroid pregnenolone into the Δ^4-3-ketosteroid progesterone (Luu-The *et al.*, 1989; Lorence *et al.*, 1990). Prolactin treatment of gonadotropin induced anestrous mink, resulted in a transient 3 fold increase in the abundance of 3β-HSD mRNA. This finding is consistent with a previous report from our laboratory in which it was found that 3β-HSD mRNA levels increased during CL reactivation and early post-implantation gestation in association with increasing levels of serum prolactin levels (Douglas *et al.*, 1996a). In addition, treatment of pregnant animals with bromocryptine, which prevents the pre-implantation rise in prolactin, also prevented the pre-implantation rise in 3β-HSD mRNA.

The final element in the progesterone biosynthetic pathway, which was examined in this study was the cholesterol transport protein StAR. StAR is believed to be the protein responsible for the mobilization of cholesterol from lipid stores, across the mitochondrial membrane to the site of the P450scc enzyme (reviewed by Waterman, 1995). The results of the current experiment revealed that the abundance of StAR mRNA did not differ between the prolactin treated and untreated groups over the course to the experimental period. This is consistent with observations from previous reports in which it was found that StAR mRNA levels did not vary over the course of mink gestation and that treatment of pregnant mink with bromocryptine had no effect on the abundance of StAR mRNA (Douglas *et al.*, 1996a).

In addition to studying the effects of prolactin on elements of the progesterone biosynthetic pathway, its effects on luteotropic hormone receptors were also examined. Prolactin exerts its biological effects by binding to a cell surface receptor and activating a number of intra-cellular signal transduction systems (Campbell *et al.*, 1994; Clevenger and Medaglia, 1994; Clevenger *et al.*, 1994; Rui *et al.*, 1994; Sidis and Horseman, 1994; Bellanga *et al.*, 1995). Previous reports have indicated that prolactin may be important for the regulation of its own receptor in the mink, since serum prolactin, PRLr mRNA and receptor binding were closely correlated in the mink (Douglas *et al.*, 1996b). In addition, it was found that treatment of pregnant mink with bromocryptine prevented the pre-implantation rise in PRLr mRNA levels seen in the untreated controls. Results of the current experiment support the hypothesis that prolactin regulates its own receptor, since treatment of anestrous animals in which follicular development and ovulation were induced resulted in a 5 fold increase in the abundance of PRLr mRNA over the pre-treated control. The ability of prolactin to regulate its own receptor has previously been demonstrated in a number of other species and tissues, including the rat lung (Amit *et al.*, 1985), liver (Amit *et al.*, 1985; Manni *et al.*, 1978; Barash *et al.*, 1988), and kidney (Barash *et al.*, 1986) as well

as in the liver of the Snell-dwarf mouse (Knazek *et al.*, 1977) and the testes of the golden hamster (Klemcke *et al.*, 1984; Klemcke *et al.*, 1986).

The effects of prolactin on mink LHr mRNA levels were also examined in the current experiment. The results revealed that prolactin treatment of animals in which follicular development and ovulation were induced with exogenous hormones, resulted in a 3-5 fold increase in the abundance of LHr mRNA over non-treated controls. The ability of prolactin and placental lactogens to regulate LHr binding sites (Gibori and Richards, 1978; Jammes *et al.*, 1985) and LHr mRNA (Gafvels *et al.*, 1992; Bjurulf *et al.*, 1994) has been reported in the CL of a number of species, including the mink (Douglas *et al.*, 1996b). Previously we have reported that there is a transient increase in the abundance of LHr mRNA in the mink during the time associated with CL reactivation and increasing serum prolactin levels. This transient peak could be abolished and LHr mRNA levels reduced by treatment of pregnant mink with bromocryptine (Douglas *et al.*, 1996b). The significance of the finding that prolactin stimulates an increase in the abundance of LHr mRNA in the mink is unclear, since the role of LH in the reactivation, maintenance and function of the mink CL has not been clearly defined. For example, there are several reports in the literature which suggest that although LH may be required for the maintenance of the mink CL post-implantation (Murphy *et al.*, 1993), it is not required for the reactivation of the mink CL (Murphy *et al.*, 1980; Murphy *et al.*, 1981; Murphy *et al.*, 1993). However, changes in the abundance of LHr mRNA during CL reactivation (Douglas *et al.*, 1996b) suggest that LH may indeed play a role in the termination of embryonic diapause in the mink.

In conclusion, it is important to note that although follicular development and ovulation were induced in the current experiment, the resulting CL failed to produce significant levels of progesterone. In addition, treatment with prolactin which induced changes in the progesterone biosynthetic elements and luteotropic hormone receptors consistent with normal CL function in the mink, also failed to stimulate significant progesterone production. This result suggests that another factor or factors in addition to prolactin may be required for normal CL function in the mink.

5.7 ACKNOWLEDGMENTS

We would like to thank Drs. T. Wise, F. Labrie and D. Stocco for the generous provision of cDNA probes for porcine P450scc, human 3β-HSD and mouse StAR. We would also like to thank Mr. M. Morrow for the care of the animals used in this experiment. Technical assistance was generously provided by Mrs. M. Dobias Goff. Funding was provided by a NSERC and MRC grants awarded to BDM.

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5.9 STATEMENT OF PARTICIPATION

Experimental Design - Deborah Douglas, Bruce Murphy

Animal Handling - Deborah Douglas, Jianhua Song

Sample Collection - Deborah Douglas, Jianhua Song

Northern and Slot Blot Analyses - Deborah Douglas

Progesterone Assay - Jianhua Song

Statistical Analysis - Deborah Douglas

Interpretation of Results - Deborah Douglas, Bruce Murphy

Paper - Deborah Douglas

5.10 CONNECTOR

Previously in Papers #1, 2 and 3 the effects of prolactin on luteotropic hormone receptors and steroidogenic elements were reported. These experiments used whole animal model systems. Changes in the abundance of mRNA for the various receptors and steroidogenic elements were determined by Northern or slot blot analysis of total RNA isolated from whole ovaries. To determine if the differences seen in these experiments were due to changes in the abundance of mRNA in the CL and not some other component of the ovary, an *in vitro* luteal cell culture system was developed.

EFFECTS OF PROLACTIN ON PRIMARY CULTURES OF LUTEAL CELLS AND AN OVARIAN TUMOR CELL LINE FROM THE MINK (*Mustela vison*).

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Key Words: Mink, embryonic diapause, prolactin, P450scc, 3β-HSD, StAR, PRLr, LHr.

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6.2 ABSTRACT

Prolactin is the principal luteotropic hormone in the mink and is essential for corpus luteum reactivation, maintenance and function. The mechanism by which it exerts its effects are unclear. The objective of this study was to monitor the effects of prolactin on the abundance of P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), steroidogenic acute regulatory protein (StAR) and luteotropic hormone receptor mRNA in primary cultures of mink luteal cells and a mink ovarian tumor cell line. For primary cell cultures, corpora lutea were collected from mink during the delay phase (March 20), CL reactivation (March 27) and the peri-implantation period of mink gestation (April 5). The luteal cells were dispersed by enzymatic digestion and placed in culture. The tumor cell line was obtained by the enzymatic dispersion of an ovarian tumor collected from a pregnant mink during embryonic diapause. In both cases, 48 hours after plating, the medium was changed and the cells were incubated for a further 12 hours with increasing doses of ovine prolactin (0, 20, 200 ng/ml). At the end of the incubation period, medium was collected to determine progesterone levels and the cells were collected from the delay period and the tumor cell line for RNA purification and slot blot analysis. Under the conditions employed in this experiment, prolactin did not stimulate progesterone production in the delay, reactivated or peri-implantation luteal cell cultures. In addition, prolactin had no significant effect on the abundance of either P450scc or PRLr mRNA. Prolactin at 200 ng/ml stimulated a significant ($P>0.05$) increase in the abundance of 3β-HSD (2.5 fold) and LHr mRNA (2.5 fold). It also caused a 35-40% decrease in the abundance of StAR mRNA. Progesterone levels from the media of the tumor cell line were below the sensitivity of the assay and treatment with prolactin had no significant effect. Low levels of P450scc, 3β-HSD and StAR mRNA were detected however, prolactin treatment had no effect on their abundance. Messenger RNA for PRLr and LHr were also detected in the ovarian cell line and prolactin treatment stimulated a significant ($P>0.05$) increase in the abundance of both PRLr and LHr mRNA. The results of the current experiments suggest that the mechanism by which prolactin induces the reactivation of the mink CL and stimulates progesterone production is not through a direct effect on steroidogenesis, since prolactin alone was unable to stimulate progesterone production, nor to alter the abundance of mRNA for the rate limiting steroidogenic enzyme P450scc and had a negative effect on the mitochondrial cholesterol transport protein StAR. The current experiment does not rule out the possibility that prolactin may work synergistically with other factors such as LH, and/or FSH, to stimulate progesterone production. This hypothesis is supported by the fact that prolactin treatment increased LHr mRNA abundance.

6.3 INTRODUCTION

Gestation in the mink is characterized by an obligate period of embryonic diapause (Hansson, 1947, Enders, 1952), during which time the mink corpus luteum secretes only low levels of progesterone (Moller, 1973). It is only after the vernal equinox on March 21 that the mink CL is reactivated and begins to produce progesterone (Allais and Martinet, 1978; Pilbeam *et al.*, 1979). The mink, as with a number of other mustelids including the ferret (Murphy, 1979) and the spotted skunk (Mead, 1975), requires an intact hypophysis for normal luteal function (Murphy and Moger, 1977; Murphy *et al.*, 1980). The pituitary is necessary not only for luteal reactivation and implantation (Murphy and Moger, 1977) but is also required for luteal maintenance after implantation (Murphy *et al.*, 1980). A number of studies have determined that the pituitary hormone, prolactin, is the principal luteotropin in the mink and is essential for the reactivation of the CL, termination of embryonic diapause and normal CL function post-implantation (Papke *et al.*, 1980; Martinet *et al.*, 1981; Murphy *et al.*, 1981). The mechanisms by which prolactin exerts its luteotropic effects remain unknown.

The steroidogenic pathway by which progesterone is synthesized in mammals has been well documented. The principal enzymes involved in luteal progesterone production are cytochrome P450scc and 3β-HSD (reviewed by Miller, 1988; Hinshelwood *et al.*, 1993). Cytochrome P450scc, along with its electron donors adrenodoxin and adrenodoxin reductase are responsible for the 20-hydroxylation, 22-hydroxylation and cleavage of the C20-C22 bond of cholesterol to produce pregnenolone and isocaproic acid. Subsequently the oxidation and isomeration of the Δ^5-3β-hydroxysteroid pregnenolone into the Δ^4-3-ketosteroid progesterone is carried out by 3β-HSD (reviewed by Miller, 1988; Hinshelwood *et al.*, 1993).

The quantity of progesterone synthesized by the CL or ovary is dependent not only on the activities of specific steroidogenic enzymes, but also upon the provision of substrate for *de novo* cholesterol synthesis, cholesterol ester stores and low density lipoproteins (LDL; reviewed by Murphy and Silavin, 1989; Gore-Langton and Armstrong, 1988). Mobilization of cholesterol from lipid stores to the vicinity of P450scc enzyme on the inner mitochondrial membrane is essential in ligand regulated steroid biosynthesis (Waterman, 1995). The mitochondrial protein StAR, which has recently been purified and cloned by Clark *et al.* (1994), has been proposed to be an important cholesterol transport protein.

The nucleic acid and deduced amino acid sequences for the genes encoding P450scc, 3β-HSD and StAR have been determined for a number of different species (P450scc; bovine: Morohashi *et al.*, 1984; human: Chung *et al.*, 1986; rat: Oonk *et al.*, 1989; porcine: Mulheron *et al.*, 1989), (3β-HSD; human placenta: Luu-The *et al.*, 1989;

bovine: Zhao *et al.*, 1989; rat: Zhao *et al.*, 1990 and 1991; macaque: Simard *et al.*, 1991; mouse: Bain *et al.*, 1991), (StAR; mouse: Clark *et al.*, 1994; human: Sugawara *et al.*, 1995). Thus, heterologous probes are available for the determination of the abundance of mRNA for these genes in different tissues under various experimental conditions in mink. In addition, our laboratory has developed homologous probes for the mink prolactin and LH receptors (Douglas *et al.*, 1996b). The objectives of this study were 1). to determine if prolactin has a direct effect on progesterone biosynthesis by monitoring changes in the response of mink luteal cells in culture to increasing doses of prolactin and 2). to determine the suitability of the mink ovarian tumor cell line as a model for studying the luteotropic effects of prolactin on the mink ovary.

6.4 MATERIALS AND METHODS

6.4.1 PRIMARY LUTEAL CELL CULTURE

Adult female mink were maintained on a commercial mink ranch (Morrow Fourrures, St Paul d'Abbotsford, PQ). They received a standard wet mink ration and water *ad libidum*. Beginning on March 3, female mink were exposed to males every two days until mated. Seven to nine days later the females were remated to different males. Successful matings were determined by the presence of motile sperm in vaginal smears.

In order to determine the effects of prolactin on mink luteal cell cultures, ovaries from pregnant mink were collected during embryonic diapause (March 22), CL reactivation (March 27) or during the peri-implantation period (April 2). The ovaries were washed twice in ice cold phosphate buffered saline (PBS), placed in Minimal Essential Medium (MEM: Gibco/BRL, Burlington, ON) and kept on ice. Corpora lutea were dissected free of interstitial tissue, pooled, weighed and minced. The cells were dispersed by enzymatic digestion with collagenase Type II (Sigma, St Louis, MO) at a concentration of 32 mg/g of tissue then washed three times in MEM and resuspended in Opti-MEM reduced serum medium (Gibco/BRL) containing 5% heat inactivated fetal calf serum (FCS: Gibco/BRL), 2.5 ml/l Fungizone (Gibco/BRL) and 5.0 ml/l penicillin-streptomycin (Gibco/BRL). The cells were plated at a concentration of 1×10^5 cells per ml, in Falcon 6 well culture plates (5 ml/well, Becton Dickinson, Lincoln Park, NJ) and incubated in 95% humidified air with 5% CO_2 at 37 C. After 48 hours the medium was changed and the cells were treated with 0, 20, 200 ng/ml ovine prolactin (Sigma). In addition, all cultured cells received 25 µg/ml 25-hydroxycholesterol (Sigma) so that they would not be substrate limited. The cultures were terminated after 12 further hours of incubation. An aliquot of medium was collected to determine progesterone concentrations and the remaining medium was discarded. The cells were lysed in 4 M guanidium isothiocyanate (GITC: Gibco/BRL) plus 0.12 M 2

mercaptoethanol (Sigma), collected and stored at -70 C until RNA extraction. The cells from two wells were collected at the end of the incubation period to determine total cell protein concentrations (Lowry *et al.*, 1951).

6.4.2 OVARIAN CELL LINE CULTURE

An ovarian tumor was removed from a two year old pregnant female mink in the delay phase of gestation. The tumor was minced and the cells were dispersed by enzymatic digestion as outlined above. The cells were passaged several times in Opti-MEM plus 5% FCS before being used for experimental purposes. Experimental culture conditions were identical to those of the primary cell cultures, except that in addition to prolactin treatment, the ovarian cell line was also treated with increasing concentrations (0, 30, 300 μM) of dibutyryl adenosine 3', 5'-monophosphate ($(Bu)_2$cAMP: Sigma). At the end of the experiment the medium was collected from each well to determine progesterone levels and the cells were collected for RNA purification and slot blot analysis.

6.4.3 SLOT BLOT ANALYSIS

Tissue samples were homogenized with a PT 3000 polytron (Brinkmann, Rexdale, ON). Total luteal and ovarian cell line RNA was extracted by CsCl (Gibco/BRL) gradient ultracentrifugation as described by Chirgwin *et al.* (1979). The homogenate was layered onto a 5.7 M CsCl (Gibco/BRL) gradient and centrifuged at 32,000 rpm (174,000 g) using a SW-41 rotor (Beckman, Mississauga, ON) for 20 hours at 22 C. The RNA pellet was dissolved and precipitated twice in 0.1 volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol. After the final washing the pellet was dissolved in diethyl pyrocarbonate (DEPC: Sigma)-treated distilled water and stored at -70 C. The RNA concentration for each sample was determined by spectrophotometric measurement at an absorbance of 260 nm.

Slot blot analysis was utilized to determine changes in the abundance of mRNA in the current experiment. Previous studies in our laboratory demonstrated the presence of single mRNA transcripts for both P450scc and 3β-HSD (Douglas *et al.*, 1996a), and multiple transcripts for StAR (Douglas *et al.*, 1996a), PRLr and LHr (Douglas *et al.*, 1996b). No indication of differential expression of mRNA transcripts was detected in these previous experiments. For slot blot analysis, 6 μg of total RNA in 50 μl of DEPC-treated water was heated to 65 C for 10 minutes with 150 μl of denaturing solution (98 μl de ionized formamide, 32 μl 37% formaldehyde and 20 μl of 10 x MOPS (0.2 M 3-[N-morpholino] propanesulphonic acid, 0.5 M sodium acetate pH 7.0, 0.01 M sodium EDTA)). An equal volume of ice-cold 20 x SCC (1 x SSC = 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) was added to the samples, which were applied to a nylon membrane (Hybond-N:

Amersham, Oakville, ON) using a slot blot manifold (BioRad, Mississauga, ON). The RNA was cross-linked to the membrane using GS gene linker UV chamber (BioRad).

Prior to hybridization the membranes were rinsed in 5 x SSPE (1 x SSPE = 180 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) then prehybridizied for 30 minutes at 60-65 C in hybridizing solution (5 x SSPE, 5 x Denhardt's solution: 0.5 % sodium dodecyl sulfate (SDS), 10% dextran sulfate). Denatured herring sperm DNA (Sigma) at a concentration of 10 μg/ml was then added to the hybridizing solution and the pre-hybridization period continued for 3 hours. Homologous (PRLr: Douglas *et al.*, 1996b; LHr: Douglas *et al.*, 1996b) and heterologous cDNA probes (porcine P450scc: Mulheron *et al.*, 1989; human 3β-HSD: Luu-The *et al.*, 1989; mouse StAR: Clark *et al.*, 1994) were labeled by random primer extension (Boehringer Mannheim, Laval, PQ) with [^{32}P]-dCTP (Dupont, Mississauga, ON). Once labeled, probes were denatured and added to the hybridizing solution. The incubation was continued overnight at 60-65 C. Membranes were washed twice, 15 minutes per wash, with 2 x SSPE plus 0.1% SDS at room temperature and twice at 60 C. The membranes were sealed in hybridizing bags with a small amount of 5 x SSPE and exposed to Kodak XAR-5 film (Rochester, NY) in cassettes with intensifying screens. All membranes were rehybridized with a human ribosomal 28S probe (Gonzales *et al.*, 1985). Autoradiographic images were digitized and analyzed using Collage software. Results are expressed as a ratio between the mRNA of interest and ribosomal 28S. This ratio was then normalized to 1, using untreated cultured cells as a control. The results were then expressed as a percent of the nontreated control.

6.4.4 PROGESTERONE ASSAY

Progesterone concentrations in the medium were determined by liquid-phase radioimmunoassay (RIA). An antiserum raised against 4-pregnen-11α-o-13,20dion hemisuccinate bovine serum albumin, kindly provided by Dr. A Goff (Lafrance and Goff, 1985) was used as the first antibody. Progesterone-11α-glucuronide-[^{125}I] iodotryamine (Amersham, Oakville, ON) was used as radioactive tracer and goat anti-rabbit IgG (Prince Laboratories, Toronto, ON) as the precipitating second antibody. The sensitivity of the assay was determined to be 6 pg. The intra- and inter-assay coefficients of variation were 12.2 and 12.3%, respectively.

6.4.5 STATISTICAL ANALYSIS

Mean + SEM values were calculated for each of the parameters measured. One way analysis of variance was used to determine significant differences between the treatment groups. In the presence of significant F values, individual differences were determined using Student's t test. A value of $P<0.05$ was considered significantly different.

6.5 RESULTS

6.5.1 PRIMARY LUTEAL CELL CULTURE

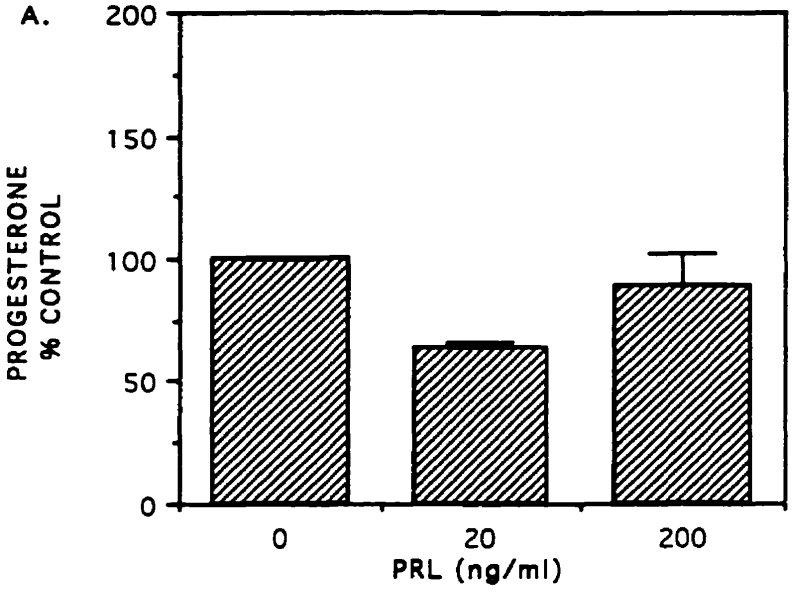
Treatment of cultured mink luteal cells collected during embryonic diapause, CL reactivation or the peri-implantation period with increasing levels of ovine prolactin had no effect on progesterone concentrations in cell medium (Figure 1). These cells were however, responsive to hormone stimulation since treatment with $(Bu)_2$cAMP increased progesterone levels 2.5 fold (data not shown). Treatment of cultured mink luteal cells with 20 or 200 ng/ml of ovine prolactin for 12 hours had no effect on the abundance of P450scc mRNA (Figure 2) but significantly ($P<0.05$) altered the abundance of both 3β-HSD (Figure 3) and StAR (Figure 4) mRNA. The abundance of 3β-HSD mRNA increased over nontreated controls in a dose dependent manner, with a 2 fold increase at 20 ng/ml and a 2.5 fold increase at 200 ng/ml (Figure 3). Prolactin significantly ($P<0.05$) decreased the abundance of StAR mRNA by 35-40% (Figure 4) at both treatment levels. In addition, prolactin treatment stimulated a dose dependent increase in the abundance of LHr mRNA (Figure 5) but had no effect on the abundance of mRNA for its own receptor (Figure 6).

6.5.2 OVARIAN CELL LINE CULTURE

Dispersion and culture (5 passages) of the ovarian tumor resulted in the survival of 2 morphologically distinct cell types. The first was epitheloid in shape (Figure 7a), while the second appeared more fibroblastic (Figure 7b). For the purposes of these preliminary experiments the two cell types were not separated. Progesterone levels in the media collected from the culture of the ovarian cell line were below the sensitivity (6 pg/ml) of the assay employed here. Treatment of the cells with increasing concentrations of prolactin or $(Bu)_2$cAMP did not result in the production of detectable amounts of progesterone. The abundance of mRNA for the steroidogenic enzymes P450scc and 3β-HSD and the cholesterol transport protein StAR were very low in the ovarian cell line and prolactin or $(Bu)_2$cAMP treatment had no effect on their abundance. Messenger RNA for prolactin and LH receptor were found in the ovarian cell line and both responded to prolactin treatment. Treatment of the ovarian cell line with 20 and 200 ng/ml of ovine prolactin resulted in a 2 fold increase in the abundance of prolactin receptor mRNA (Figure 8a). In addition,

treatment of the cell line with 200 ng/ml of prolactin resulted in a 2 fold increase in LHr mRNA (Figure 8b).

Figure 1. (a). Progesterone concentrations (mean + SEM) in media from mink luteal cell cultures collected during embryonic diapause (March 22) and treated with increasing concentrations of ovine prolactin. Values are expressed as a percentage of the untreated control. Progesterone concentrations were determined by double antibody RIA. (b). Progesterone concentration (mean + SEM) in media from mink luteal cells collected during CL reactivation. (c). Progesterone concentration (mean + SEM) in media from mink luteal cells collected during the peri-implantation period.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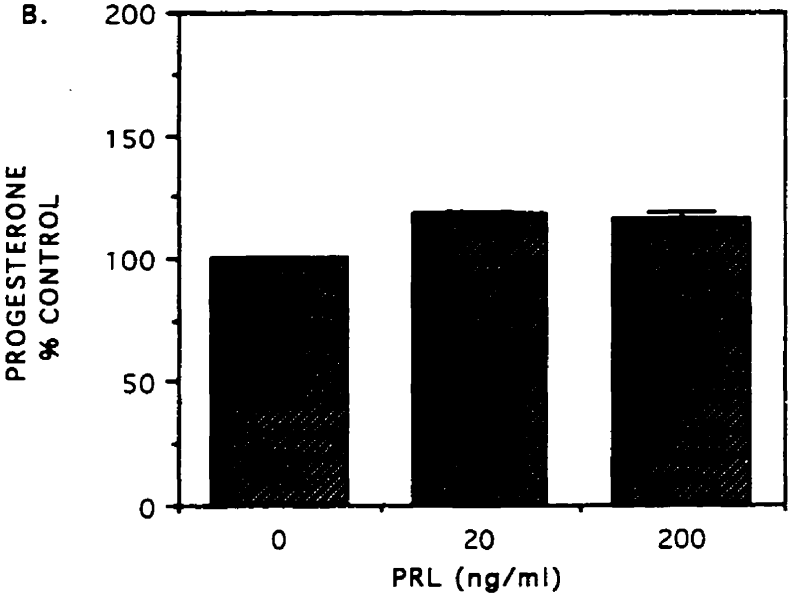
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200
PRL (ng/ml)

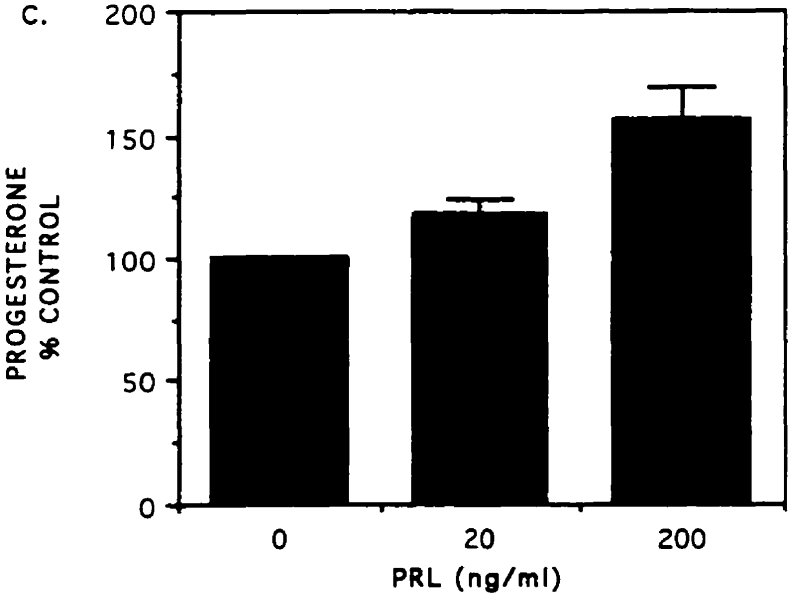
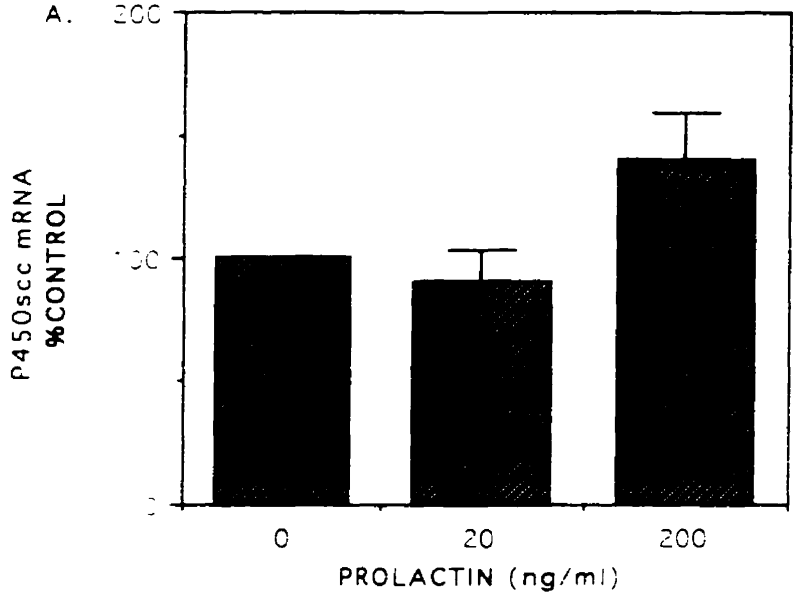

C.
200
150
100
50
0
PROGESTERONE
% CONTROL
0
20
200
PRL (ng/ml)

Figure 2. (a). Abundance of P450scc mRNA (mean + SEM) in mink luteal cells collected during embryonic diapause and treated with increasing concentrations of ovine prolactin. Values are expressed as a percentage of the untreated control. (b). Autoradiograph of slot blot labeled with P450scc. (c). Same autoradiograph re-labeled with human ribosomal 28S.


A.
200
100
0
P450scc mRNA
%CONTROL
0
20
200
PROLACTIN (ng/ml)


B.
P450scc

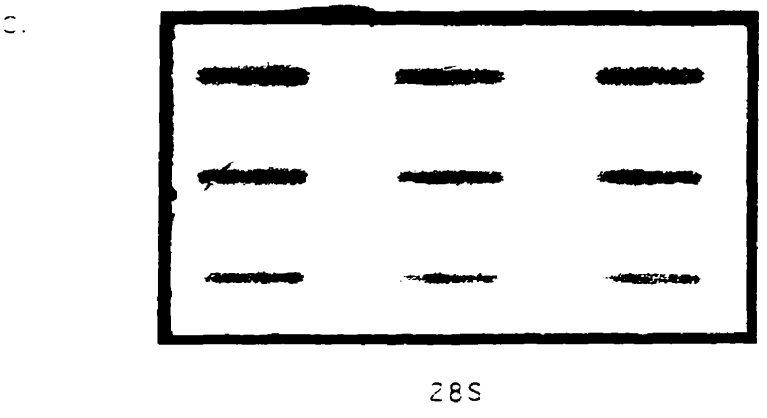
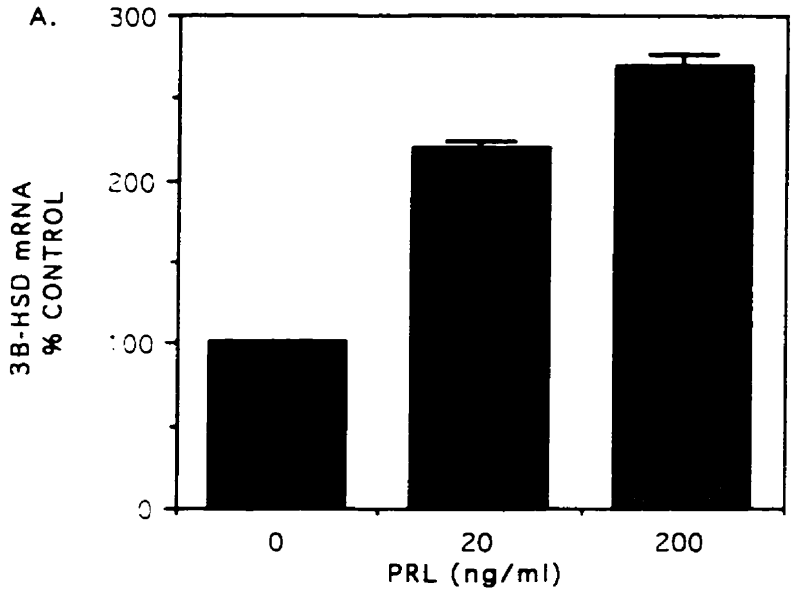
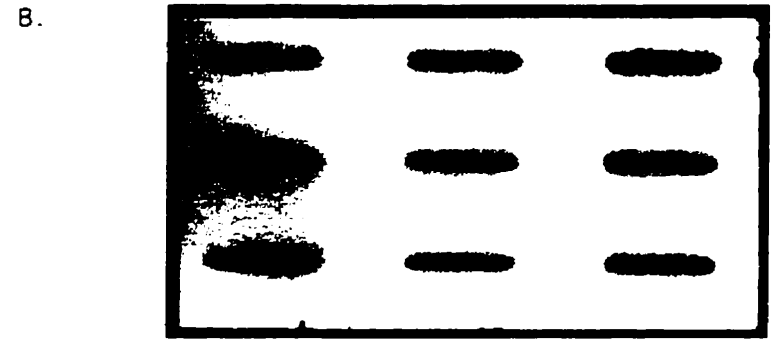

C.
28S

Figure 3. (a). Abundance of 3β-HSD mRNA (mean + SEM) in mink luteal cells collected during embryonic diapause and treated with increasing concentrations of ovine prolactin. Values are expressed as a percentage of the untreated control. (b). Autoradiograph of slot blot labeled with 3β-HSD. (c). Same autoradiograph re-labeled with human ribosomal 28S.


A.
300
200
100
0
3B-HSD mRNA
% CONTROL
0
20
200
PRL (ng/ml)


B.
3B-HSD

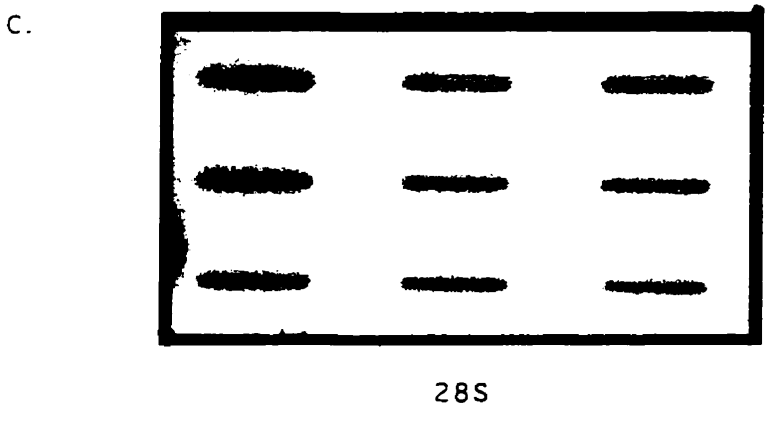
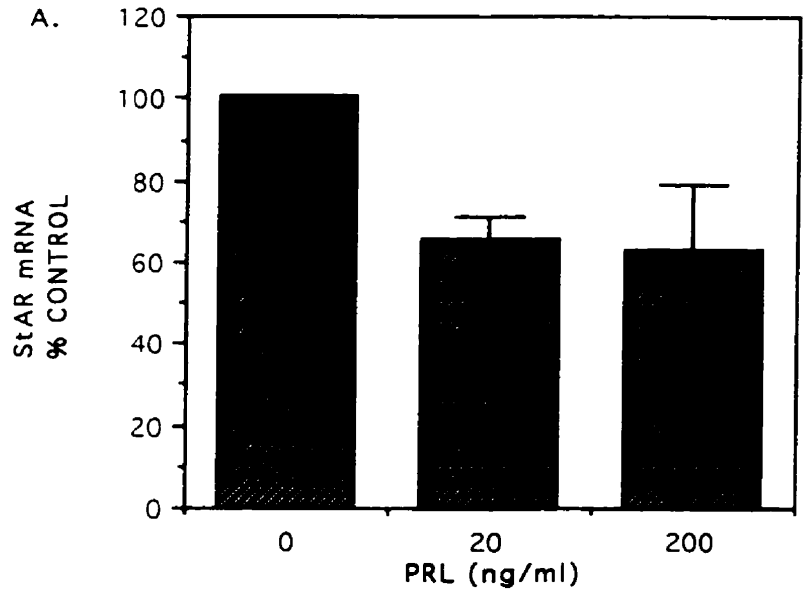
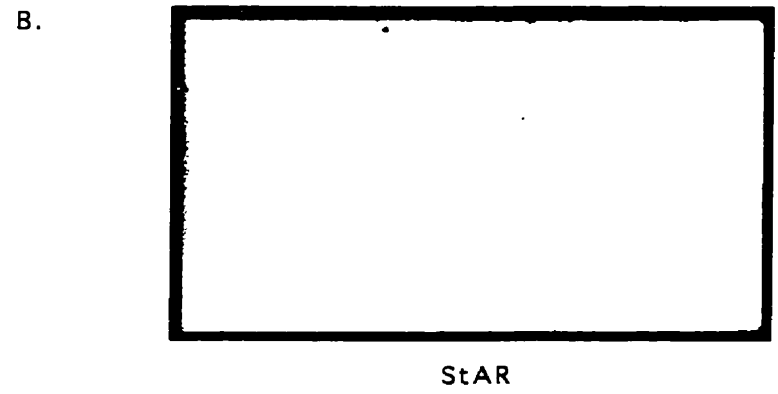

C.
28S

Figure 4. (a). Abundance of StAR mRNA (mean + SEM) in mink luteal cells collected during embryonic diapause and treated with increasing concentrations of ovine prolactin. Values are expressed as a percentage of the untreated control. (b). Autoradiograph of slot blot labeled with StAR. (c). Same autoradiograph re-labeled with human ribosomal 28S.


A.
120
100
80
60
40
20
0
StAR mRNA
% CONTROL
0
20
200
PRL (ng/ml)


B.
StAR

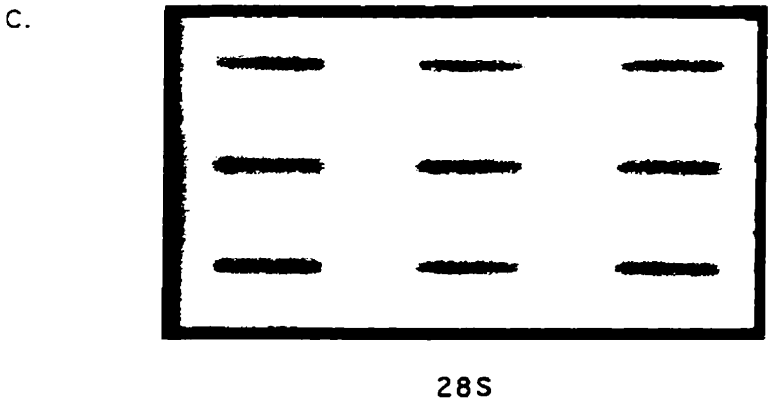
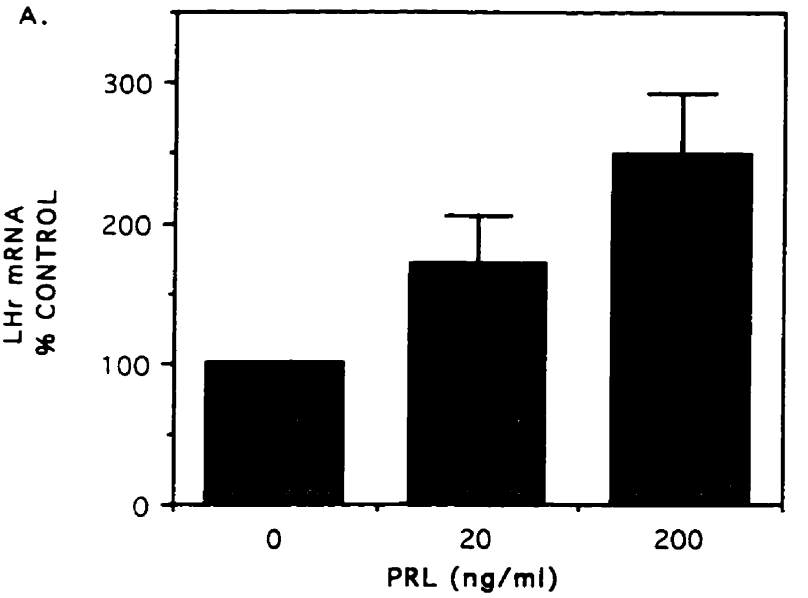
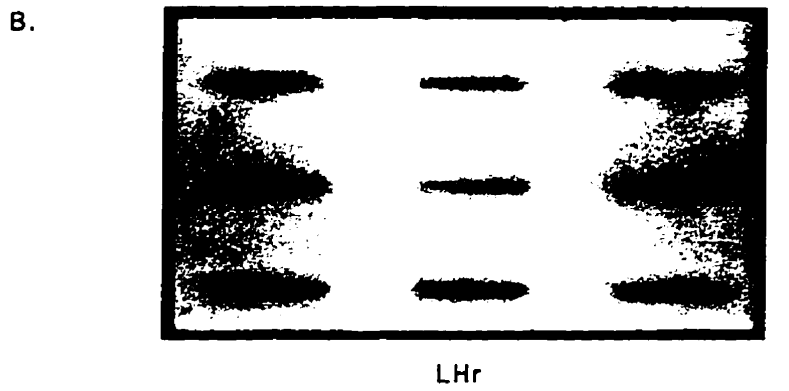

C.
28S

Figure 5. (a). Abundance of LHr mRNA (mean + SEM) in mink luteal cells collected during embryonic diapause and treated with increasing concentrations of ovine prolactin. Values are expressed as a percentage of the untreated control. (b). Autoradiograph of slot blot labeled with LHr. (c). Same autoradiograph re-labeled with human ribosomal 28S.


A.
300
200
100
0
LHr mRNA
% CONTROL
0
20
200
PRL (ng/ml)


B.
LHr

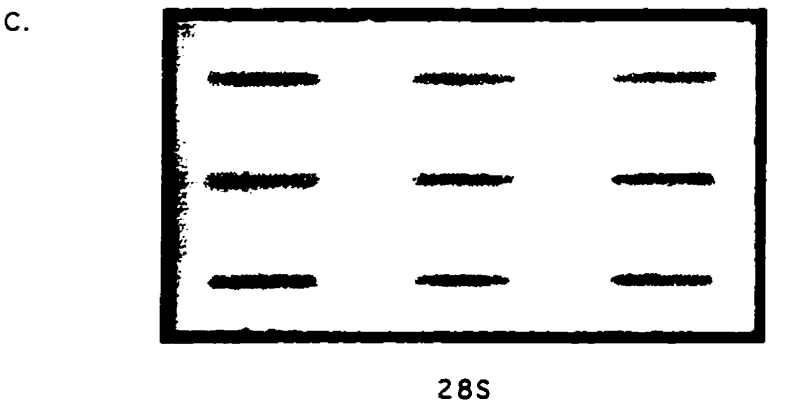
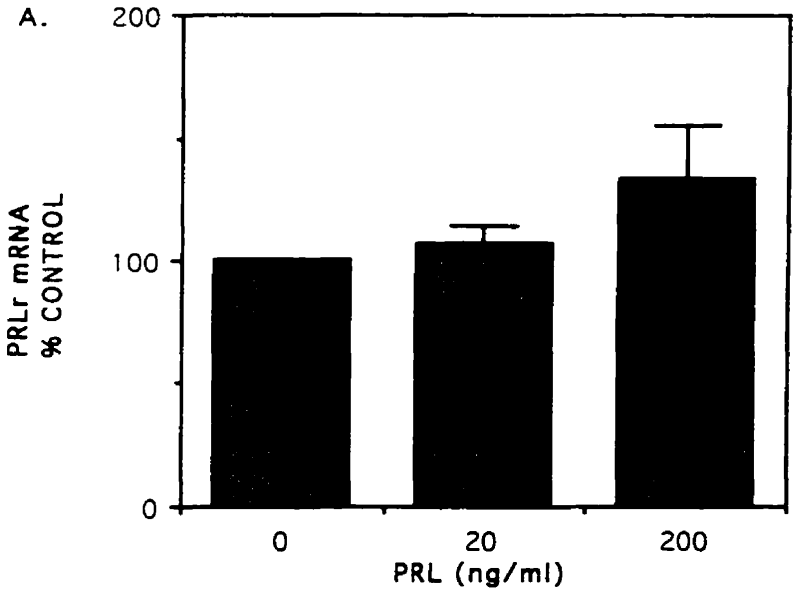
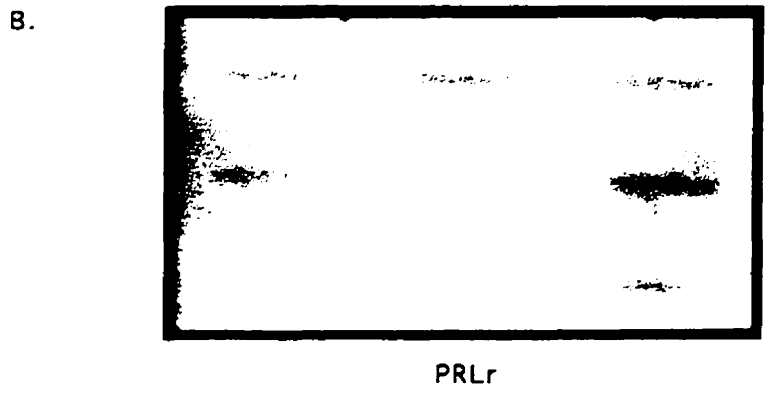

C.
28S

Figure 6. (a). Abundance of PRLr mRNA (mean + SEM) in mink luteal cells collected during embryonic diapause and treated with increasing concentrations of ovine prolactin. Values are expressed as a percentage of the untreated control. (b). Autoradiograph of slot blot labeled with PRLr. (c). Same autoradiograph re-labeled with human ribosomal 28S.


A.
200
100
0
PRLr mRNA
% CONTROL
0
20
200
PRL (ng/ml)


B.
PRLr

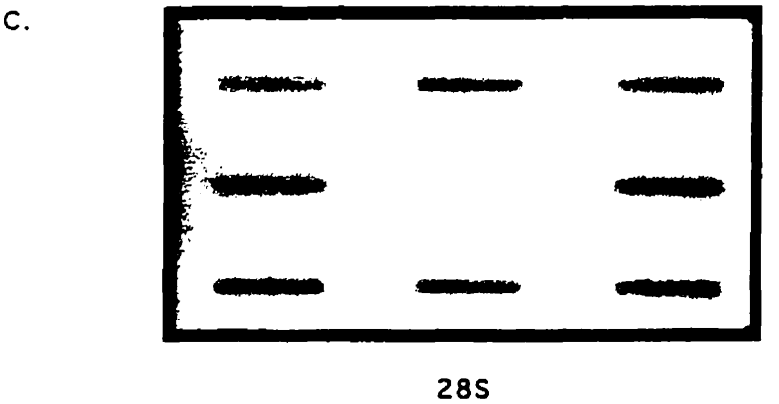
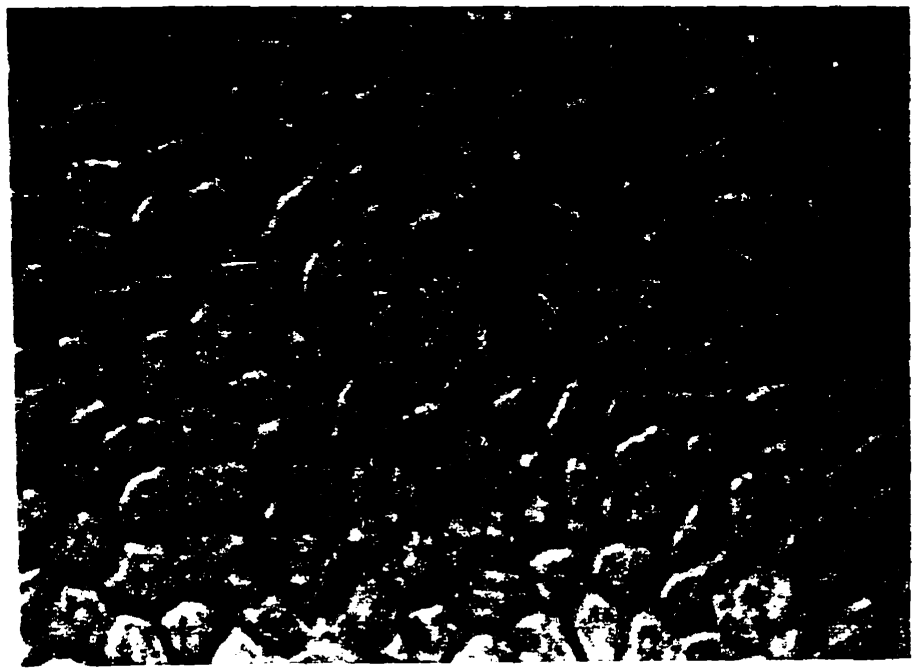

C.
28S

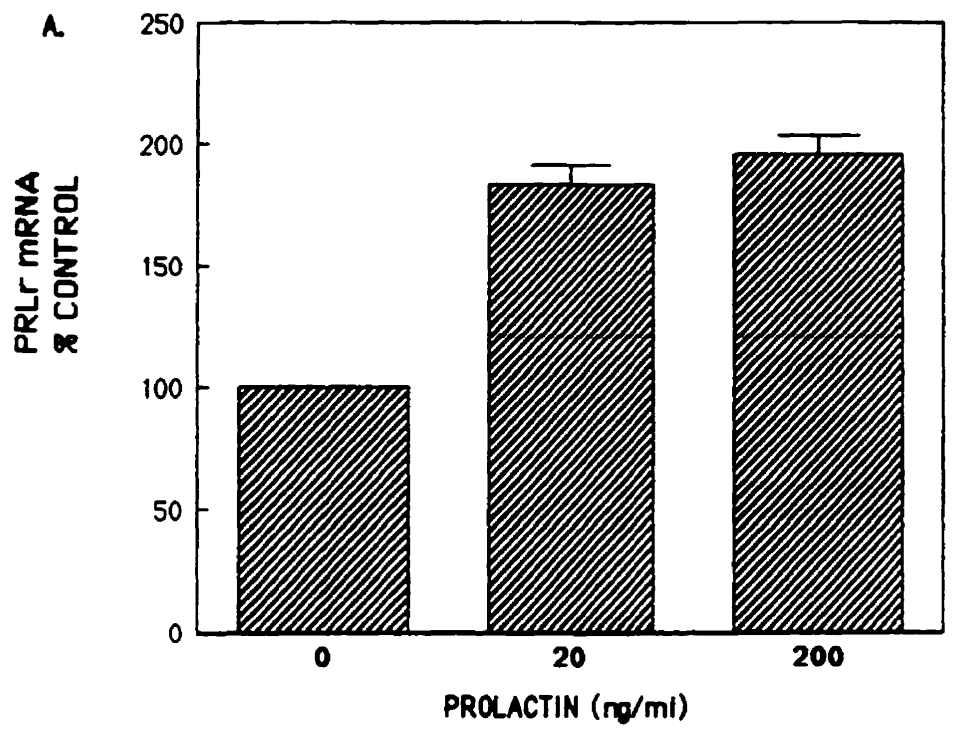
Figure 7. (a). Photomicrograph (x 100) of the epitheloid cell type from the mink ovarian tumor isolated from a pregnant mink in the delay phase of gestation. (b). Photomicrograph (x 100) of the fibroblastic cell type from the mink ovarian tumor.

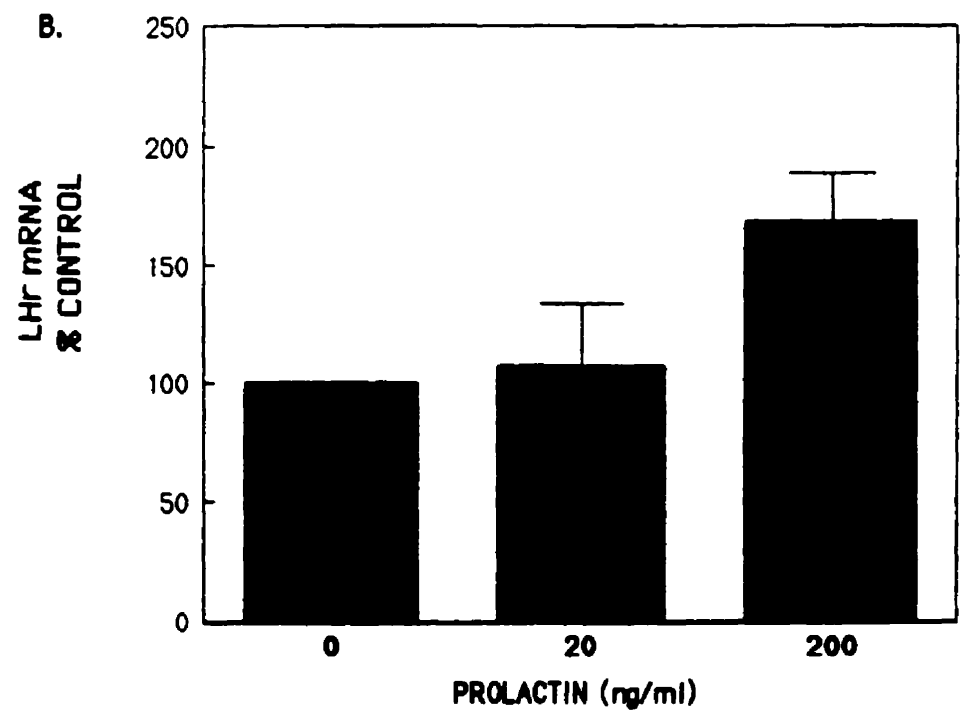
A.



B.

Figure 8. (a). Abundance of PRLr and (b). LHr mRNA (mean + SEM) in the mink ovarian tumor cell line treated with increasing concentrations of ovine prolactin. Values are expressed as a percentage of the untreated control.


A.
PRLr mRNA
% CONTROL
250
200
150
100
50
0
0
20
200
PROLACTIN (ng/ml)


B.
LHr mRNA
% CONTROL
250
200
150
100
50
0
0
20
200
PROLACTIN (ng/ml)

6.6 DISCUSSION

Prolactin is the principal luteotropic hormone in the mink and is required not only for the reactivation of the delay CL but also for its maintenance and function post-implantation (Papke *et al.*, 1980; Martinet *et al.*, 1981; Murphy *et al.*, 1981). The role of prolactin in maintaining CL function in other species has been attributed to indirect effects through the induction and maintenance of LH receptors (Gafvelds *et al.*, 1992; Bjurulf *et al.*, 1994), the inhibition of progesterone degradation (Eckstein and Nimrod, 1979; Jones *et al.*, 1983; Albarracin *et al.*, 1994) or through direct effects via a luteotropic influence on the steroidogenic pathway (reviewed by Murphy and Rajkumar, 1985). The mechanism(s) by which prolactin exerts its effects in the mink are unclear. The current experiment was undertaken to determine the effects of prolactin on both the progesterone biosynthetic pathway and the abundance of LHr mRNA in mink luteal cells.

Results of the current experiment revealed that prolactin alone was unable to stimulate progesterone production in cultured mink luteal cells collected at different times during gestation. This is in agreement with an earlier report by Stoufflet *et al.* (1989) in which ovine prolactin (500 ng/ml) had no effect on progesterone production in cultured mink luteal cells after 2 hours of incubation, regardless of the stage of pregnancy from which they were collected. The ferret, a closely related species to the mink, which is also dependent on prolactin for luteal function, also showed no effect of prolactin on progesterone production by cultured luteal cells during the peri-implantation stage of gestation (McKibbin *et al.*, 1984). However, the results of the current experiment are in contrast to a study by Murphy *et al.* (1993) in which they reported a small (<11%) but significant increase in progesterone production by mink luteal cells in response to as little as 1 ng/ml of ovine prolactin, although the response was not dose dependent (1 to 1000 ng/ml).

Cytochrome P450scc is the mitochondrial enzyme which together with adrenodoxin and adrenodoxin reductase (Hanukoglu and Hanukoglu, 1986) is responsible for the conversion of cholesterol to pregnenolone, which is a rate limiting step in progesterone biosynthesis (reviewed by Miller, 1988; Hinshelwood *et al.*, 1993). Results from the current experiment revealed that prolactin has no effect on the abundance of P450scc mRNA in cultured mink luteal cells collected during embryonic diapause. This contrasts with a study by Oonk *et al.* (1990) in which it was reported that luteinized, but not preovulatory, rat granulosa cells respond to prolactin both *in vivo* and *in vitro* by increases in P450scc mRNA. However, the results of the present study are in agreement with previous investigations in our laboratory which revealed that the abundance of P450scc mRNA was not influenced by endogenous prolactin levels *in vivo*. In that study, P450scc

mRNA levels did not vary significantly over the course of mink gestation and were not affected by treatment which suppressed endogenous prolactin levels (Douglas *et al.*, 1996a). In addition, exogenous prolactin had an inhibitory effect on the abundance of P450scc mRNA of mink in which follicular development, ovulation and CL function had been induced in anestrous animals (Douglas *et al.*, 1996c). Although prolactin had a negative effect on the abundance of P450scc mRNA in the present experiments, these findings do not rule out the possibility that prolactin may exert effects on P450scc at either the protein or enzyme activity level. Goldring *et al.* (1987) found that, although the LH surge in the rat caused a rapid increase in P450scc mRNA abundance, the protein content for this enzyme remained largely unchanged unless animals were also treated with prolactin.

In the current experiment, prolactin clearly increased the abundance of 3β-HSD mRNA, the enzyme responsible for the oxidation and isomerization of pregnenolone to progesterone (reviewed by Miller, 1988; Hinshelwood *et al.*, 1993). This result is consistent with previous experiments in our laboratory. An experiment conducted *in vivo* showed that ovarian 3β-HSD mRNA levels increased during the pre-implantation stage of mink gestation (Douglas *et al.*, 1996a). This increase could be abolished by treatment of animals with bromocryptine, which suppresses endogenous prolactin levels (Douglas *et al.*, 1996a). In addition, prolactin treatment of mink bearing CL resulted in a transient 6 fold increase in 3β-HSD mRNA levels (Douglas *et al.*, 1996d). These three lines of evidence indicate that prolactin can stimulate increases in the abundance of 3β-HSD mRNA. Although, 3β-HSD is not a rate limiting step in the biosynthesis of steroids, the elevation in this enzyme may reflect changes in the direction of steroidogenesis from the Δ5 to the Δ4 pathway with the resultant production of progesterone. This finding is consistent with the so called permissive effect of prolactin on progesterone production i.e. prolactin does not directly stimulate progesterone rather it maintains the biosynthetic machinery which allows other factors to induce progesterone production.

StAR is an acutely regulated mitochondrial protein involved in the transport of cholesterol across the mitochondrial membrane to the site of P450scc enzyme activity (Clark *et al.*, 1994; Lin *et al.*, 1995). Treatment of cultured mink luteal cells with either 20 or 200 ng/ml of ovine prolactin decreased the abundance of StAR mRNA. This is in contrast to previous studies *in vivo*, in which alterations of endogenous prolactin levels had no effect on the abundance of StAR mRNA (Douglas *et al.*, 1996a and 1996d). Analysis of serum progesterone and prolactin profiles in this species have demonstrated that during late gestation the progesterone levels decline while prolactin levels continue to increase (Martinet *et al.*, 1982). In addition, long term treatment of intact mink with prolactin has been shown to reduce circulating progesterone levels (Murphy *et al.*, 1990). The present

study suggests that prolactin down regulation of StAR mRNA may be a factor in the prolactin induced reduction of progesterone in late pregnancy in this species.

Douglas *et al.* (1996b) reported that changes in serum prolactin levels, PRLr mRNA and PRLr binding were temporally correlated in the mink ovary throughout gestation. Therefore, it was suggested that prolactin may regulate its own receptor, especially since increases in PRLr mRNA associated with CL reactivation and implantation could be abolished by treatment with the dopamine agonist bromocryptine. Studies in other species including the rat liver (Posner *et al.*, 1975; Barash *et al.*, 1988) have shown that prolactin induces its own receptor. In the current experiment, prolactin alone had no effect on the abundance of PRLr mRNA in cultured mink luteal cells. However, this may reflect the short duration of treatment or the requirement of other factors present in the intact ovary.

Prolactin stimulated increases in the abundance of LHr mRNA in the current experiment. Previous experiments have shown that there was a transient increase in LHr mRNA associated with the time of CL reactivation and that treatment of animals with bromocryptine could prevent this peak (Douglas *et al.*, 1996b). In addition, treatment of animals *in vivo* with bromocryptine caused a reduction in the abundance of LHr mRNA below pre-treated controls (Douglas *et al.*, 1996d). It has also been shown that LH can stimulate a dose dependent increase in progesterone production in mink luteal cells *in vitro* (Murphy *et al.*, 1993; Douglas *et al.*, 1996c). Therefore, it is possible that a further effect of prolactin on progesterone biosynthesis in the mink may be to increase the sensitivity of the CL to circulating levels of LH by increasing the number of its receptors. A similar function for prolactin has been described in a number of species (Gibori and Richards, 1978; Gafvelds *et al.*, 1992; Bjurulf *et al.*, 1994; Yuan *et al.*, 1995).

The mink ovarian tumor cell line isolated in this experiment was investigated as a possible model for studying the effects of prolactin on the mink ovary. The results of the current experiment revealed that under the culture conditions employed here the tumor cell line did not produce detectable amounts of progesterone. Neither treatment with prolactin nor cAMP, which has been shown to stimulate a 2.5 fold increase in progesterone levels in cultured mink luteal cells (Douglas *et al.*, 1996c), had any effect on progesterone production in these cells. The low level of progesterone produced by these cells is probably due to the low abundance of mRNA for the steroidogenic elements P450scc and StAR, since both of these elements are important rate limiting steps in progesterone biosynthesis. In addition, these cells had an altered response to prolactin, since prolactin had no effect on the abundance of 3β-HSD mRNA.

Although, the tumor cell line did not produce detectable levels of progesterone, it did contain mRNA for both LHr and PRLr and responded to prolactin treatment by increasing the abundance of mRNA for these receptors. Therefore, this cell line may be

useful for determining the mechanism by which prolactin is able to regulate luteotropic hormone receptors in the mink. For many years the intra-cellular signal transduction system by which prolactin exerted its biological effect was unknown. Recently, however, numerous transduction systems have been shown to function with prolactin (Campbell *et al.*, 1994; Clevenger and Medaglia, 1994; Clevenger *et al.*, 1994; Rui *et al.*, 1994; Sidis and Horseman, 1994; Bellanga *et al.*, 1995).

In conclusion, the results of this experiment and others in our laboratory do not support the hypothesis that prolactin has a direct effect on the progesterone biosynthesis in the mink (Douglas *et al.* 1996a and 1996d). Prolactin alone was incapable of altering progesterone production in cultured mink luteal cells regardless of the stage of gestation from which they were collected. In addition, prolactin had no effect on the important rate limiting enzyme P450scc and had a negative effect on the message for the acutely regulated protein, StAR. However, prolactin stimulated increases in the abundance of 3β-HSD mRNA and induced LHr mRNA. Together these observations suggest a supportive rather than direct role for prolactin in the maintenance and function of the mink CL .

6.7 ACKNOWLEDGMENTS

We would like to thank Drs. T. Wise, F. Labrie and D. Stocco for the generous provision of cDNA probes for porcine P450scc, human 3β-HSD and mouse StAR. We would also like to thank Mr. M. Morrow for the care of the animals used in this experiment. Technical assistance was generously provided by Mrs. M. Dobias Goff. Funding was provided by a NSERC and MRC grants awarded to BDM.

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6.9 STATEMENT OF PARTICIPATION

Experimental Design - Deborah Douglas, Bruce Murphy

Animal Handling - Deborah Douglas, Jianhua Song, G Moreau

Sample Collection - Deborah Douglas, Jianhua Song, G Moreau

Tissue Culture - Deborah Douglas

Slot Blot Analyses - Deborah Douglas

Progesterone Assay - G Moreau

Statistical Analysis - Deborah Douglas

Interpretation of Results - Deborah Douglas, Bruce Murphy

Paper - Deborah Douglas

6.10 CONNECTOR

Prolactin is the principal luteotropic hormone in the mink and is necessary for normal CL function throughout gestation. Results from Paper #1 revealed that in addition to prolactin, the abundance of mRNA for another luteotropic hormone LH varied around the time of CL reactivation and could be altered by changing the endogenous levels of prolactin. In addition, Paper #3 showed that although follicular development and ovulation could be induced in anestrous mink and prolactin treatment could stimulate changes in luteotropic hormone receptors and steroidogenic elements, prolactin alone was unable to stimulate progesterone production. Therefore, the effects of other gonadotropic hormones on luteal function in the mink were determined using an *in vitro* model system.

EFFECTS OF GONADOTROPIC HORMONES ON P450 SIDE CHAIN CLEAVAGE (P450scc), 3β-HYDROXYSTEROID DEHYDROGENASE (3β-HSD) AND STEROIDOGENIC ACUTE REGULATORY PROTEIN (StAR) mRNA IN CULTURED MINK (*Mustela vison*) LUTEAL CELLS.

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Key Words: Mink, embryonic diapause, LH, FSH, $(Bu)_2$cAMP, P450scc, 3β-HSD, StAR.

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7.2 ABSTRACT

Prolactin is the principal luteotropic hormone in the mink. The role of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the reactivation, maintenance and function of the mink corpus luteum (CL) is unclear. The objective of this study was to monitor changes in abundance of the mRNA for several elements essential to progesterone biosynthesis namely P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD) and steroidogenic acute regulatory protein (StAR) in cultured mink luteal cells. Cells were treated with LH, FSH and dibutyryl adenosine 3', 5'-monophosphate ($(Bu)_2$cAMP). Corpora lutea were collected from mink during embryonic diapause (March 20 to 22) or the peri-implantation period of mink gestation. The luteal cells were dispersed by enzymatic digestion and placed in culture. After 48 hours the media were changed and the luteal cells were incubated for a further 12 hours with increasing doses of porcine LH (0, 20, 200 ng/ml), porcine FSH (0, 20, 200 ng/ml) or $(Bu)_2$cAMP (0, 30, 300 μM). At the end of the incubation period, an aliquot of medium was collected to determine progesterone levels and the cells were collected for RNA purification and slot blot analysis. Under the culture conditions employed in this experiment, treatment of mink luteal cells with LH stimulated significant ($P<0.05$) increases in progesterone production in cultures from the delay and peri-implantation ovaries while FSH treatment stimulated an increase only in the delay culture. Luteinizing hormone had no effect on the abundance of 3β-HSD or StAR mRNA but significantly ($P<0.05$) increased the abundance of P450scc mRNA (2.5 fold) at the 200 ng/ml level. Follicle stimulating hormone had no effect on the abundance of either P450scc or StAR mRNA but increased 3β-HSD mRNA significantly ($P<0.05$) at the 20 ng/ml dose. Treatment of mink luteal cells with $(Bu)_2$cAMP stimulated the greatest increase in progesterone production of all the hormone treatments (2.5 fold) in both the delay and peri-implantation cultures. In addition, it stimulated significant ($P<0.05$) increases in the abundance of P450scc (2 fold), 3β-HSD (2.5 fold) and StAR (3 fold) mRNA. In conclusion, treatment of cultured mink luteal cells with LH, FSH and $(Bu)_2$cAMP stimulated increases in progesterone production. In addition, these hormones were able to stimulate changes in the abundance of mRNA for the important steroidogenic enzymes P450scc and 3β-HSD and also for the mitochondrial protein StAR. These results indicate that LH and /or FSH may play a role in the reactivation, maintenance and function of the mink CL through the stimulation of progesterone biosynthesis.

7.3 INTRODUCTION

Gestation in the mink is characterized by an obligate period of embryonic diapause (Hansson, 1947, Enders, 1952). During the delay phase the mink corpus luteum secretes low levels of progesterone (Moller, 1973). It is only after the vernal equinox that the mink CL is reactivated and begins to produce elevated quantities of progesterone (Allais and Martinet, 1978; Pilbeam *et al.*, 1979). Mustelids including the mink (Murphy and Moger, 1977; Murphy *et al.*, 1980), ferret (Murphy, 1979) and the spotted skunk (Mead, 1975), require an intact hypophysis for normal luteal function. The pituitary is necessary not only for luteal reactivation and implantation (Murphy and Moger, 1977) but is also required for luteal maintenance after implantation (Murphy *et al.*, 1980). A number of studies have determined that the pituitary hormone prolactin is the principal luteotropin in the mink and is essential for the reactivation of the CL, termination of embryonic diapause and normal CL function post-implantation (Papke *et al.*, 1980; Martinet *et al.*, 1981; Murphy *et al.*, 1981). The mechanism(s) by which prolactin exerts its luteotropic effects are unknown. Luteinizing hormone and FSH are other luteotropic hormones secreted by the pituitary. In some rodent species, complexes of prolactin, LH and/or FSH are required for normal CL function (rat: Morishige and Rothchild, 1974; hamster: Yuan and Greenwald, 1994a and 1994b). The role of LH and FSH in the function of the mink CL has not been clearly defined. Previous studies suggest that they are not essential for CL reactivation (Murphy *et al.*, 1981), but may play a role in the maintenance of the post-implantation CL (Murphy *et al.*, 1993). However, recent studies from our laboratory suggest that one of the functions of prolactin in the mink is to regulate the abundance of LHr mRNA in the corpus luteum during the period of CL reactivation (Douglas *et al.*, 1996b and 1996c). This in turn indicates that LH may play a role in the reactivation process.

The enzymes which bring about luteal progesterone production are cytochrome P450scc and 3β-HSD (reviewed by Miller, 1988; Hinshelwood *et al.*, 1993). Cytochrome P450scc, along with its electron donors adrenodoxin and adrenodoxin reductase are responsible for the 20-hydroxylation, 22-hydroxylation and cleavage of the C20-C22 bond of cholesterol to produce pregnenolone and isocaproic acid. Subsequently the oxidation and isomeration of the Δ^5-3β-hydroxysteroid pregnenolone into the Δ^4-3-ketosteroid progesterone is carried out by 3β-HSD (reviewed by Miller, 1988; Hinshelwood *et al.*, 1993).

The amount of progesterone produced by the CL or ovary is not only dependent on the activities of specific steroidogenic enzymes but also upon the provision of cholesterol as substrate from *de novo* synthesis, cholesterol ester stores and low density lipoproteins (LDL; reviewed by Gore-Langton and Armstrong, 1988; Murphy and Silavin, 1984).

Mobilization of cholesterol from lipid stores to the vicinity of P450scc enzyme on the inner mitochondrial membrane is essential in ligand regulated steroid biosynthesis (Waterman, 1995). The mitochondrial protein StAR, recently purified and for which the gene sequence has been derived by Clark *et al.* (1994), has been proposed to be important in the delivery of cholesterol across the mitochondrial membrane.

The nucleic acid and deduced amino acid sequences for the genes encoding P450scc, 3β-HSD and StAR have been determined for a number of species (P450scc; bovine: Morohashi *et al.*, 1984; human: Chung *et al.*, 1986; rat: Oonk *et al.*, 1989; porcine: Mulheron *et al.*, 1989), (3β-HSD; human placenta: Luu-The *et al.*, 1989; bovine: Zhao *et al.*, 1989; rat: Zhao *et al.*, 1990; Zhao *et al.*, 1991; macaque: Simard *et al.*, 1991; mouse: Bain *et al.*, 1991), (StAR; mouse: Clark *et al.*, 1994; human: Sugawara *et al.*, 1995; ovine: Juengel *et al.*, 1995). Thus, heterologous cDNA probes are available to determine the abundance of mRNA for these genes in different tissues under various experimental conditions. The main objectives of this study were to determine if LH, FSH or $(Bu)_2$cAMP had any effects on progesterone biosynthesis in cultured mink luteal cells in the absence of prolactin stimulation and to determine if they may play a role in CL reactivation in the mink.

7.4 MATERIALS AND METHODS

7.4.1 LUTEAL CELL CULTURE

Adult female mink were maintained on a commercial mink ranch (Morrow Fourrures, St Paul d'Abbotsford, PQ). They received a standard wet mink ration and water *ad libitum*. Mating followed standard farm procedure. Beginning on March 3, the female mink were exposed to males every two days until mated. Seven to nine days later the females were remated to different males. Successful matings were determined by the presence of motile sperm in vaginal smears.

In order to determine the effects of gonadotropic hormones on mink luteal cell cultures, ovaries from pregnant mink were collected during embryonic diapause (March 20 and March 22) or during the peri-implantation period (April 2 and April 5). The ovaries were washed twice in ice cold phosphate buffered saline (PBS), placed in Minimal Essential Medium (MEM: Gibco/BRL, Burlington, ON) and kept on ice. Corpora lutea were dissected free of interstitial tissue, pooled, weighed and minced. The cells were dispersed by enzymatic digestion with collagenase Type II (Sigma, St Louis, MO) at a concentration of 32 mg per g of tissue. The cells were washed 3 times in MEM and resuspended in Opti-MEM reduced serum medium (Gibco/BRL) containing 5% heat inactivated fetal calf serum (FCS: Gibco/BRL), 2.5 ml/l Fungizone (Gibco/BRL) and 5.0 ml/l penicillin-streptomycin (Gibco/BRL). The cells were plated at a concentration of $1x10^5$ cells per ml, in Falcon 6

well culture plates (5 ml/well, Becton Dickinson, Lincoln Park, NJ) and incubated in 95% humidified air with 5% CO_2 at 37 C. After 48 hours the medium was changed and the cells were treated with either 0, 20, 200 ng/ml porcine LH (NIH USDA-pLH-B-1, Beltsville, MD), 0, 20 or 200 ng/ml of porcine FSH (Sigma) or 0, 30, 300 μM $(Bu)_2$cAMP (3 replicates/treatment). In addition, all cultured cells received 25 μg/ml 25-hydroxycholesterol (Sigma) so that they would not be substrate limited. The cultures were terminated after 12 hours of incubation. An aliquot of medium was collected to determine progesterone concentrations, the remaining medium was discarded. The cells were lysed in 4 M guanidium isothiocyanate (GITC: Gibco/BRL) plus 0.12 M 2 mercaptoethanol (Sigma), collected and stored at -70 C until RNA extraction. The cells from two representative wells were collected at the end of the incubation period to determine total cell protein concentrations (Lowry *et al.*, 1951).

7.4.2 RNA PURIFICATION AND SLOT BLOT ANALYSIS

Total luteal cell RNA was extracted by CsCl (Gibco/BRL) gradient ultracentrifugation as described by Chirgwin *et al.* (1979). Tissue samples were homogenized with a PT 3000 polytron (Brinkmann, Rexdale, ON). The homogenate was layered onto a 5.7 M CsCl (Gibco/BRL) gradient and centrifuged at 32,000 rpm (174,000 g) using a SW-41 rotor (Beckman, Mississauga, ON) for 20 hours at 22 C. The RNA pellet was dissolved and precipitated twice in 0.1 volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol. After the final washing the pellet was dissolved in diethyl pyrocarbonate (DEPC: Sigma)-treated distilled water and stored at -70 C. The RNA concentration for each sample was determined by spectrophotometric measurement at an absorbance of 260 nm.

Slot blot analysis was utilized to determine changes in the abundance of mRNA in the current experiment. Previous studies in our laboratory demonstrated the presence of single mRNA transcripts for P450scc and 3β-HSD and multiple transcripts for StAR (Douglas *et al.*, 1996a). There was no indication of differential regulation of the StAR transcripts in the previous experiments (Douglas *et al.*, 1996a and 1996c). For slot blot analysis, 6 μg of total RNA in 50 μl of DEPC-treated water was heated to 65 C for 10 minutes with 150 μl of denaturing solution (98 μl deionized formamide, 32 μl 37% formaldehyde and 20 μl of 10 x MOPS (0.2 M 3-[N-morpholino] propanesulphonic acid, 0.5 M sodium acetate pH 7.0, 0.01 M sodium EDTA)). An equal volume of ice-cold 20 x SCC (1 x SSC = 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) was added to the samples, which were applied to a nylon membrane (Hybond-N: Amersham, Oakville, ON) using a slot blot manifold (BioRad, Mississauga, ON). The RNA was cross-linked to the membrane using GS gene linker UV chamber (BioRad).

Prior to hybridization the membranes were rinsed in 5 x SSPE (1 x SSPE = 180 mM NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.4) then prehybridizied for 30 minutes at 60-65 C in hybridizing solution (5 x SSPE, 5 x Denhardt's solution: 0.5 % sodium dodecyl sulfate (SDS), 10% dextran sulfate). Denatured herring sperm DNA (Sigma) at a concentration of 10 μg/ml was then added to the hybridizing solution and the pre-hybridization period continued for at least another 3 hours. Heterologous cDNA probes (porcine P450scc: Mulheron *et al.*, 1989; human 3β-HSD: Luu-The *et al.*, 1989; mouse StAR: Clark *et al.*, 1994) were labeled by random primer extension (Boehringer Mannheim, Laval, PQ) with [^{32}P]-dCTP (Dupont, Mississauga, ON). Labeled probes were denatured and added to the hybridizing solution. The incubation was continued overnight at 60-65 C. Membranes were washed twice, 15 minutes per wash, with 2 x SSPE plus 0.1% SDS at room temperature and twice at 60 C. The membranes were sealed in hybridizing bags with a small amount of 5 x SSPE and exposed to Kodak XAR-5 film (Rochester, NY) in cassettes with intensifying screens. All membranes were rehybridized with a human ribosomal 28S probe (Gonzales *et al.*, 1985). Autoradiographic images were digitized and analyzed using Collage software. Results are expressed as a ratio between the cDNA probe of interest and ribosomal 28S. These arbitrary units were then expressed as a percent of the nontreated control.

7.4.3 PROGESTERONE ASSAY

Medium progesterone concentrations were determined by liquid-phase radioimmunoassay (RIA). An antiserum raised against 4-pregnen-11α-o-13,20-dion-hemisuccinate bovine serum albumin, kindly provided by Dr. A Goff (Lafrance and Goff, 1985) was used as the first antibody. Progesterone-11α-glucuronide-[^{125}I] iodotryamine (Amersham, Oakville, ON) was used as radioactive tracer and goat anti-rabbit IgG (Prince Laboratories, Toronto, ON) as the precipitating second antibody. The sensitivity of the assay was determined to be 6 pg/ml. The intra- and inter-assay coefficients of variation were 12.2 and 12.3%, respectively.

7.4.4 STATISTICAL ANALYSIS

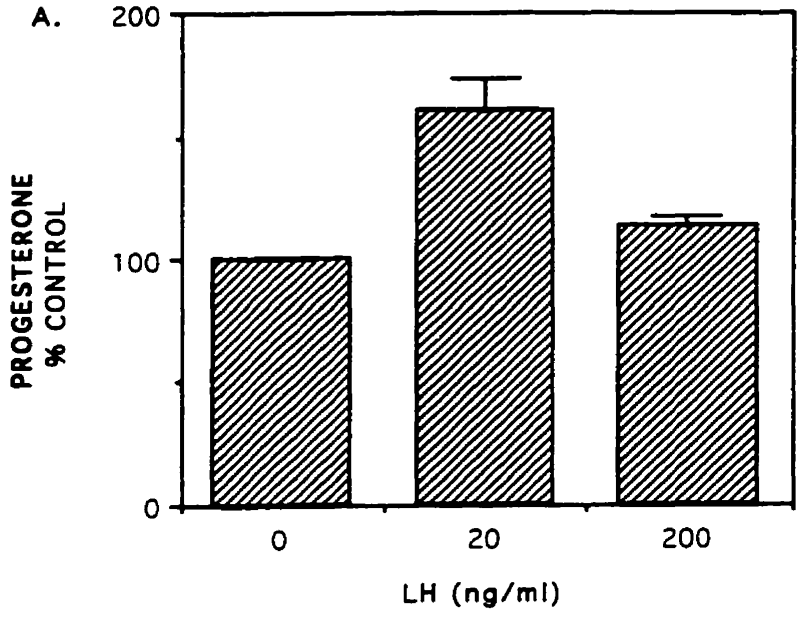
Mean + SEM values were calculated for each of the parameters measured. One way analysis of variance was used to determine significant differences between the treatment groups. In the presence of significant F values, individual differences were determined using Student's t test. A value of $P<0.05$ was chosen to discriminate significance.

7.5 RESULTS

The low dose (20 ng/ml) of porcine LH stimulated a small but significant ($P<0.05$) increase in progesterone concentrations in both the delay and peri-implantation cultures (61% and 29%; Figure 1a and 1b). The high dose (200 ng/ml) of porcine FSH also stimulated an increase (45%) in progesterone production in delay cultures (Figure 2a) although it had no effect on cells taken during the peri-implantation period (Figure 2b). Treatment of mink luteal cells with 300 μM of $(Bu)_2$cAMP stimulated the most dramatic increases in progesterone production of all the hormone treatments. Treatment of delay cells with $(Bu)_2$cAMP caused a 2.5 fold increase in progesterone levels after 12 hours of stimulation (Figure 3a), while in the peri-implantation period it caused a 71% increase (Figure 3b).

Porcine LH at either the 20 or 200 ng/ml dose had no effect on either 3β-HSD (Figure 5a) or StAR (Figure 6a) mRNA abundance but caused a dose dependent increase in P450scc mRNA (Figure 4a). Porcine FSH had no significant effect on the abundance of either P450scc (Figure 4b) or StAR (Figure 6b) mRNA but significantly ($P<0.05$) increased 3β-HSD mRNA at 20 ng/ml (Figure 5a). Treatment of cultured mink luteal cells with $(Bu)_2$cAMP had significant ($P<0.05$) effects on the abundance of P450scc, 3β-HSD and StAR mRNA. At 300 μM, $(Bu)_2$cAMP stimulated a 2 fold increase in P450scc mRNA (Figure 4c). It stimulated an increase in 3β-HSD mRNA in a dose dependent manner, with a 2.5 fold increase at the 300 mM concentration (Figure 5c). Finally, $(Bu)_2$cAMP stimulated a 3 fold increase in StAR mRNA at the 300 μM concentration but had no significant effect at 30 μM (Figure 6c).

Figure 1. (a). Progesterone concentrations (mean + SEM) in medium from mink luteal cell cultures collected during embryonic diapause and treated with increasing concentrations of porcine LH. Values are expressed as a percentage of the untreated control. Progesterone concentrations were determined by double antibody RIA. (b). Progesterone concentration (mean + SEM) in medium from mink luteal cells collected during the peri-implantation period and treated with increasing concentrations of porcine LH.


A.
200
100
0
PROGESTERONE
% CONTROL
0
20
200
LH (ng/ml)

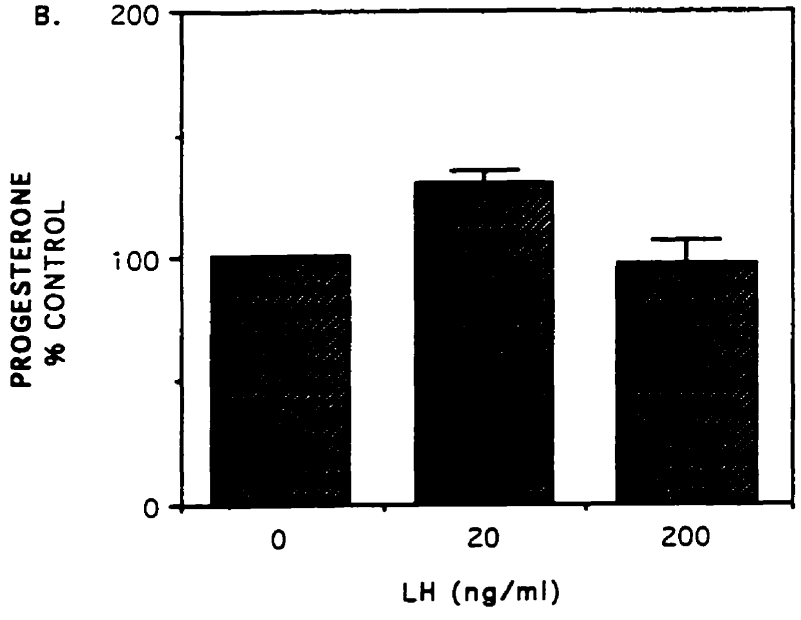
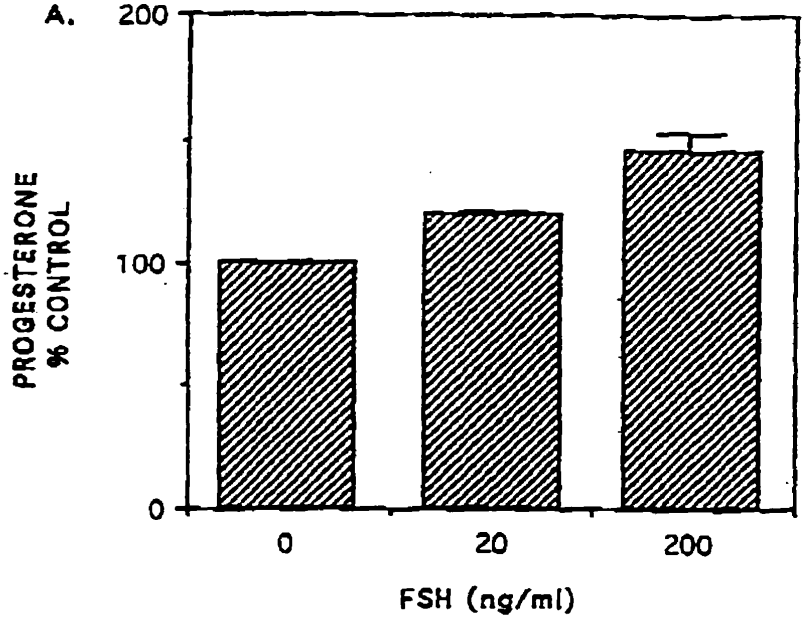

B.
200
100
0
PROGESTERONE
% CONTROL
0
20
200
LH (ng/ml)

Figure 2. (a). Progesterone concentrations (mean + SEM) in medium from mink luteal cell cultures collected during embryonic diapause and treated with increasing concentrations of porcine FSH. Values are expressed as a percentage of the untreated control. Progesterone concentrations were determined by double antibody RIA. (b). Progesterone concentration (mean + SEM) in medium from mink luteal cells collected during the peri-implantation period and treated with increasing concentrations of porcine FSH.


A.
200
100
0
PROGESTERONE
% CONTROL
0
20
200
FSH (ng/ml)

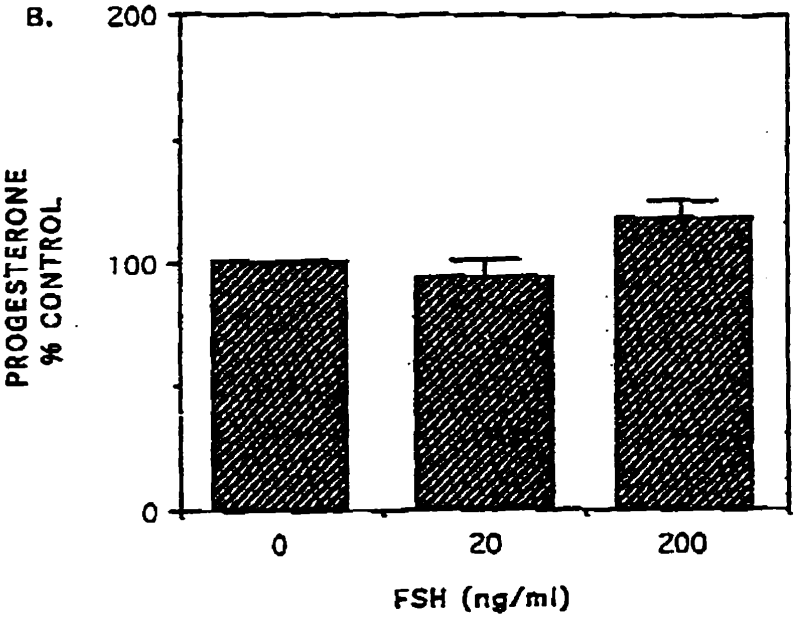
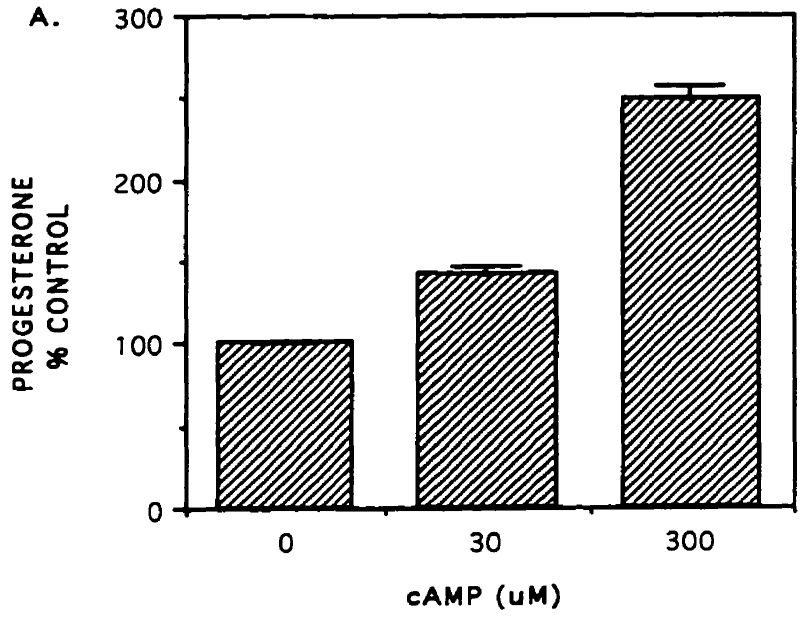

B.
200
100
0
PROGESTERONE
% CONTROL
0
20
200
FSH (ng/ml)

Figure 3. (a). Progesterone concentrations (mean + SEM) in medium from mink luteal cell cultures collected during embryonic diapause and treated with increasing concentrations of $(Bu)_2$cAMP. Values are expressed as a percentage of the untreated control. Progesterone concentrations were determined by double antibody RIA. (b). Progesterone concentration (mean + SEM) in medium from mink luteal cells collected during the peri-implantation period and treated with increasing concentrations of $(Bu)_2$cAMP.


A.
300
200
100
0
PROGESTERONE
% CONTROL
0
30
300
cAMP (uM)

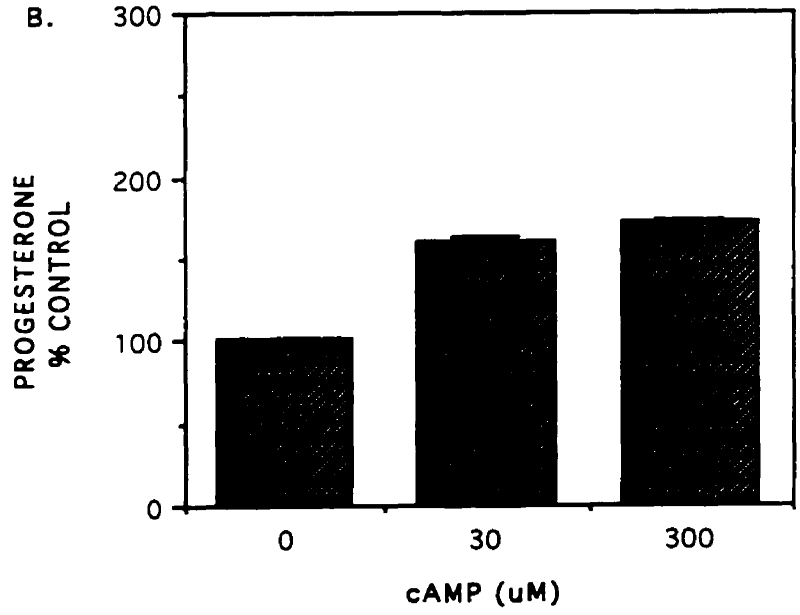

B.
300
200
100
0
PROGESTERONE
% CONTROL
0
30
300
cAMP (uM)

Figure 4. Abundance of P450scc mRNA (mean + SEM) in mink luteal cells collected during embryonic diapause and treated with increasing concentrations of (a). porcine LH, (b). porcine FSH and (c). $(Bu)_2$cAMP. Values were normalized using ribosomal 28S and are expressed as a percentage of the untreated control.

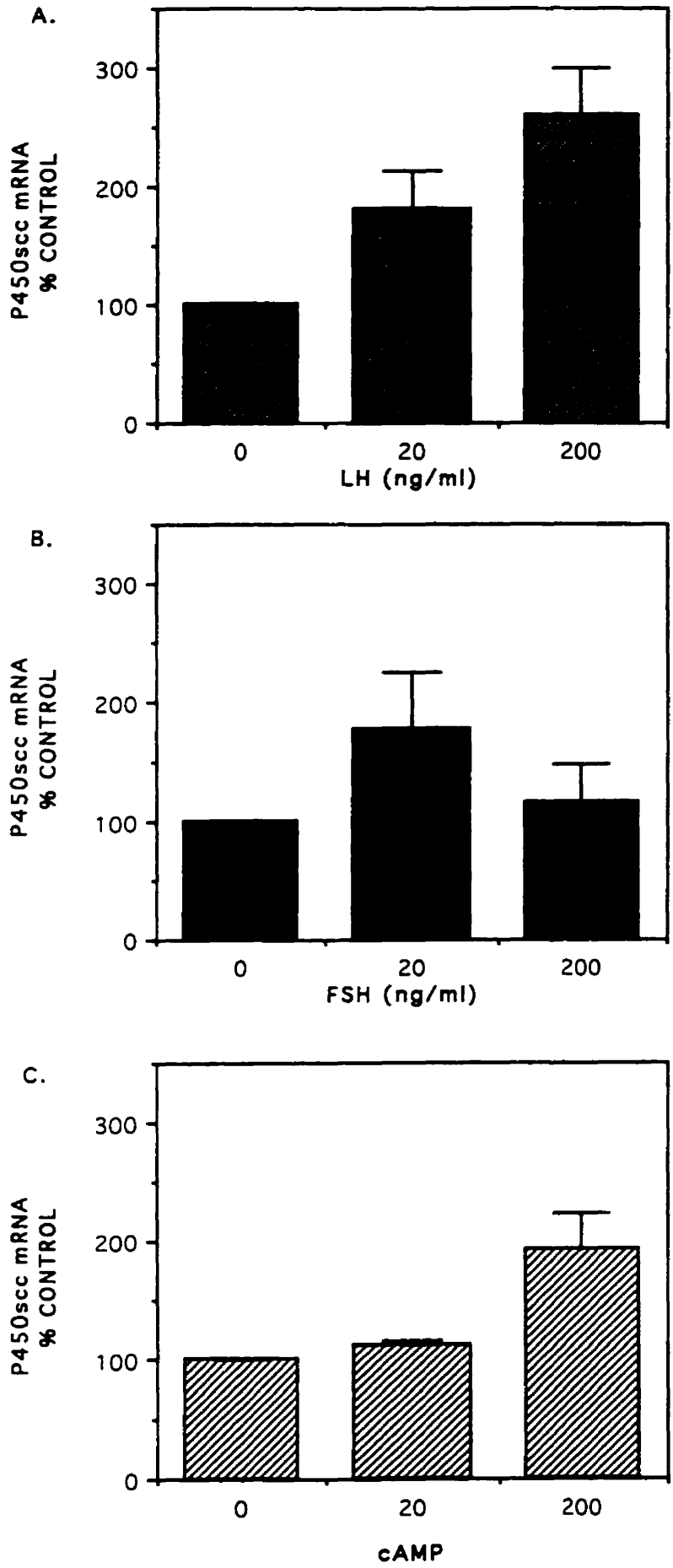

A.
P450scc mRNA
% CONTROL
300
200
100
0
0
20
200
LH (ng/ml)
B.
P450scc mRNA
% CONTROL
300
200
100
0
0
20
200
FSH (ng/ml)
C.
P450scc mRNA
% CONTROL
300
200
100
0
0
20
200
cAMP

Figure 5. Abundance of 3β-HSD mRNA (mean + SEM) in mink luteal cells collected during embryonic diapause and treated with increasing concentrations of (a). porcine LH, (b). porcine FSH and (c). $(Bu)_2$cAMP. Values were normalized using ribosomal 28S and are expressed as a percentage of the untreated control.

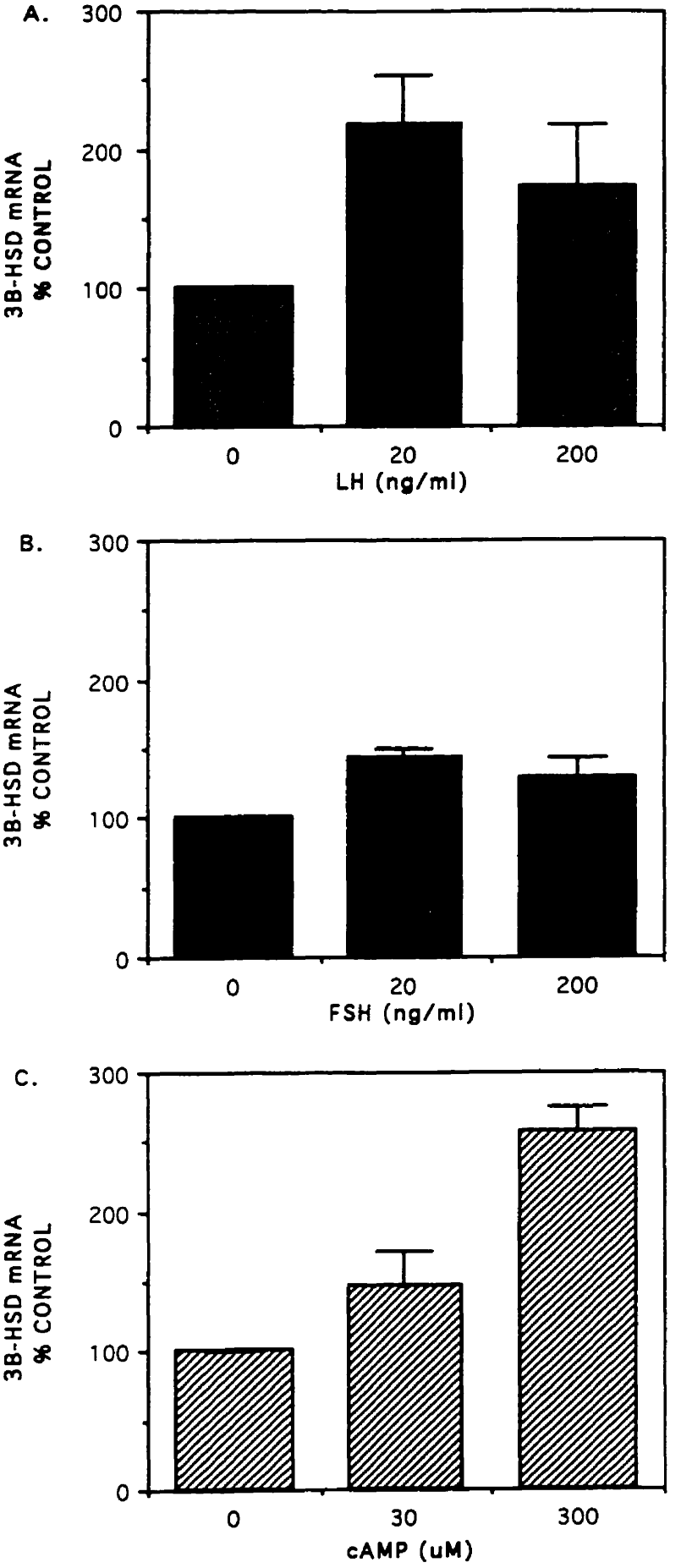
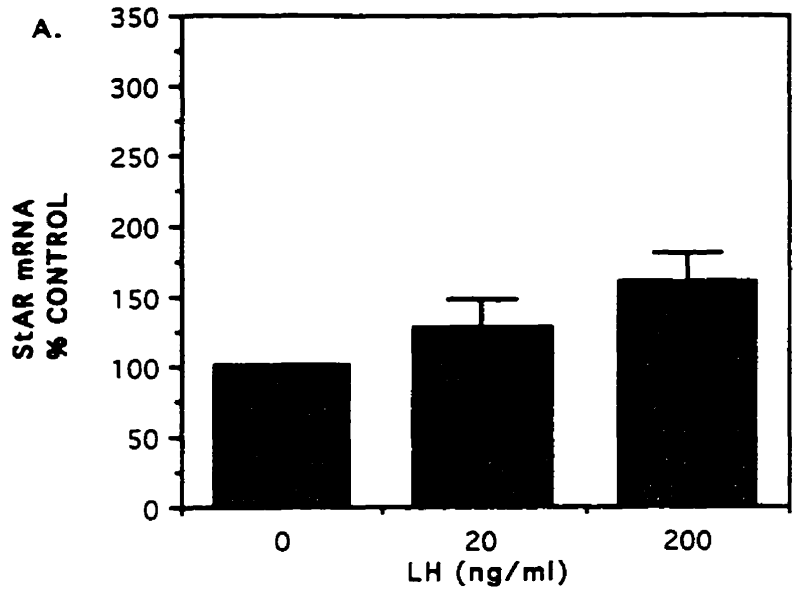
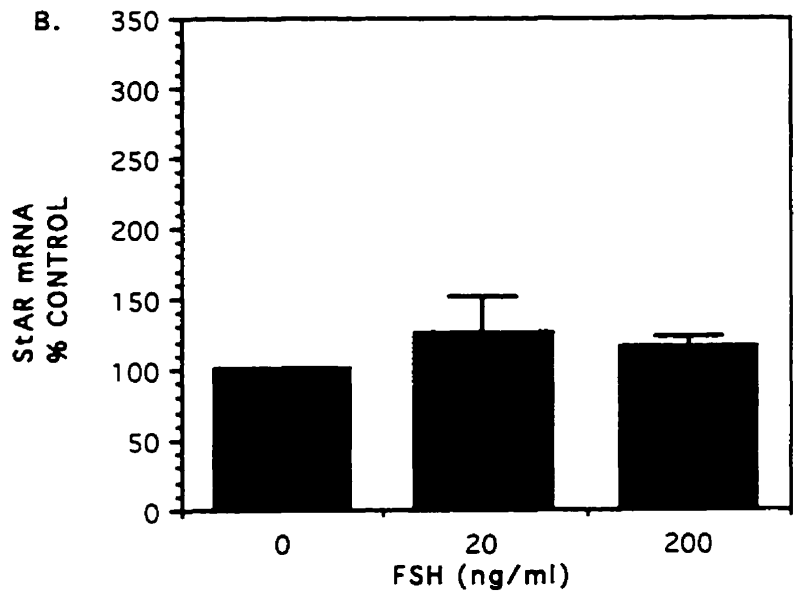
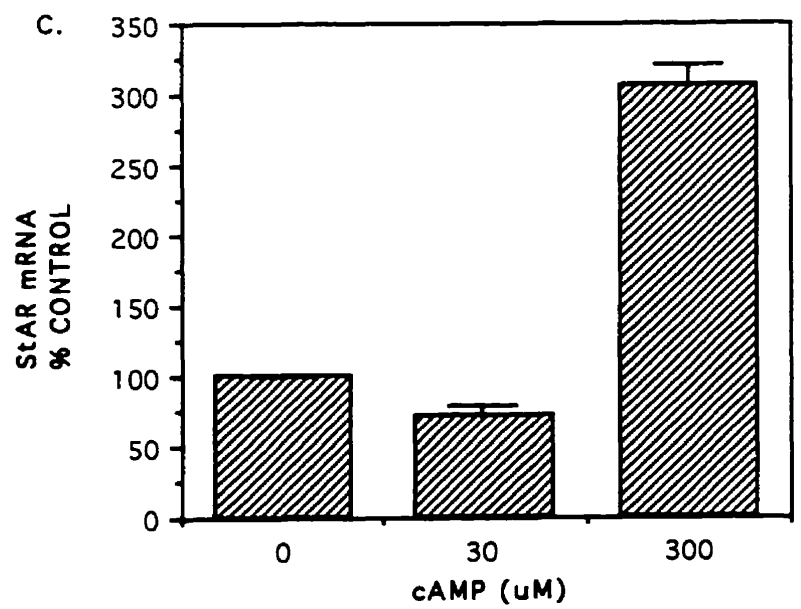

A.
300
200
100
0
3B-HSD mRNA
% CONTROL
0
20
200
LH (ng/ml)
B.
300
200
100
0
3B-HSD mRNA
% CONTROL
0
20
200
FSH (ng/ml)
C.
300
200
100
0
3B-HSD mRNA
% CONTROL
0
30
300
cAMP (uM)

Figure 6. Abundance of StAR mRNA (mean + SEM) in mink luteal cells collected during embryonic diapause and treated with increasing concentrations of (a). porcine LH, (b). porcine FSH and (c). $(Bu)_2$cAMP. Values were normalized using ribosomal 28S and are expressed as a percentage of the untreated control.


A.
StAR mRNA
% CONTROL
350
300
250
200
150
100
50
0
0
20
200
LH (ng/ml)


B.
StAR mRNA
% CONTROL
350
300
250
200
150
100
50
0
0
20
200
FSH (ng/ml)


C.
StAR mRNA
% CONTROL
350
300
250
200
150
100
50
0
0
30
300
cAMP (uM)

7.6 DISCUSSION

The actions of the gonadotropins LH and FSH are primarily mediated by the intracellular transducer cAMP. Treatment of cultured mink luteal cells, collected from ovaries in the delay phase of gestation, with the cAMP analog $(Bu)_2$cAMP resulted in a 2.5 fold increase in progesterone production. In cells collected later in gestation, around the time of implantation, $(Bu)_2$cAMP was still able to stimulate progesterone production above that of control values, although, the degree of stimulation was less then that observed in the delay cells. These results indicate that a functional cAMP signal transduction system is present in the mink during embryonic diapause and its absence is therefore not the reason why the delayed CL is unable to produce significant levels of progesterone.

Both LH and FSH stimulated modest increases in progesterone production in cultured mink luteal cells. Previous reports on the ability of LH to stimulate progesterone production in mink luteal cells are contradictory. Stoufflet *et al.* (1989) stated that LH had no significant effect on progesterone production regardless of the stage of gestation from which the luteal cells were collected. While Murphy *et al.* (1993) reported that, over a 2 hour incubation period, LH induced increases in progesterone production in cells collected from mink during the post-implantation period of pregnancy. The divergent responses of mink luteal cells to LH treatment in these experiments may be due to differences in treatment doses and the length of the incubation period. In the study by Stoufflet *et al.* (1989), luteal cells were treated with 500 ng/ml of ovine LH; however, in the current experiment only the low dose (20 ng/ml) and not the high dose (200 ng/ml) of LH stimulated progesterone production. Changes in the response of mink luteal cells to varying doses of LH were also reflected in the study by Murphy *et al.* (1993) in which they showed a dose dependent effect at lower doses of LH (1.0 and 10 ng/ml) but not at higher doses.

The effect of FSH on the accumulation of progesterone in the media was modest (45%) but significant in cultured mink luteal cells collected from animals during embryonic diapause; however, the effect of FSH on progesterone production in the peri-implantation luteal cells was not different ($P<0.05$) from controls. In other species, FSH has been found to stimulate progesterone production in granulosa (Armstrong and Dorrington, 1976; Adashi *et al.*, 1981; Gorospe and Spangelo, 1993; Moon *et al.*, 1978) and luteal cell (Gregoraszczuk, 1989; Yuan and Greenwald, 1994a and 1994b) cultures. However, the results of the current experiment are in contrast to an earlier report in the mink by Stoufflet *et al.* (1989) in which they found that 250 ng/ml of ovine FSH had no effect on progesterone production in cultured mink luteal cells regardless of the stage of gestation from which they were collected. These differences may reflect differences in culture conditions

The results of the current experiment reveal a common characteristic in the pattern of progesterone stimulated by LH, FSH, and $(Bu)_2$cAMP in cultured mink luteal cells, namely these hormones were able to stimulate larger increases in progesterone production in delay cells versus cells collected during the peri-implantation stage of mink gestation. It is important to note that basal levels of progesterone were higher in the peri-implantation cells verses delay cells (data not presented). In the case of LH the reduction in its ability to stimulate progesterone production in the peri-implantation cells may be explained by differences in LHr mRNA levels as reported by Douglas *et al.* (1996b). Differences in LHr mRNA probably reflected differences in LH receptor numbers which in turn effects the hormones ability to stimulate progesterone production. Although reduction in the number of receptors for LH and FSH may account for the reduction in their ability to stimulate progesterone production in peri-implantation luteal cells, it does not account for differences observed in $(Bu)_2$cAMP treated cells. Therefore, it is possible that mink luteal cells may lose their ability to respond to gonadotropin stimulation as gestation progresses. This observation, however, is not in agreement with an earlier report by Murphy *et al.* (1993) in which they found that treatment of mink with GnRH antibodies to reduce endogenous LH and/or FSH levels had no effect on luteal progesterone production prior to implantation but reduced progesterone levels after implantation.

Since both LH and FSH stimulated progesterone production in mink luteal cells, we determined if these effects were due to alterations in the abundance of mRNA for the enzymes involved. P450scc is responsible for the conversion of cholesterol into pregnenolone, the main substrate used in all steroidogenesis. Results of the current experiment revealed that LH and $(Bu)_2$cAMP stimulated dose dependent increases in the abundance of P450scc mRNA, while treatment of cells with FSH had no significant effect on its abundance. Numerous studies in the rat have shown that cAMP and LH mediate the induction of P450scc mRNA in granulosa cells *in vivo* (Richards *et al.*, 1979; Richards and Bogovich, 1982; Bogovich *et al.*, 1981) and *in vitro* (Richards *et al.*, 1986; Voutilainen *et al.*, 1986; Funkenstein *et al.*, 1984; Trzeciak *et al.*, 1986; Waterman and Simpson, 1985). However, P450scc mRNA expression in the rat is believed to be regulated in a sequential manner, being cAMP-dependent in granulosa cells and cAMP-independent in luteal cells (Oonk *et al.*, 1989). In primary cultures of bovine luteal cells, treatment with LH or forskolin, which elevates cAMP, resulted in elevated mRNA levels for P450scc (Simpson and Waterman, 1988; Boggaram *et al.*, 1989; Lauber *et al.*, 1991). Similarly, levels of mRNA for P450scc increased in human granulosa-lutein cells treated with forskolin (McAllister *et al.*, 1990). The constitutive expression of P450scc mRNA in the corpus luteum which has been suggested in a number of species (rat: Goldring *et al.*, 1987; Richards *et al.*, 1987; Hickey *et al.*, 1988; macaque: Simard *et al.*, 1991) appears not to be

universal (bovine: Rogers *et al.*, 1986; sheep: Belfiore *et al.*, 1994; Juengel *et al.*, 1994). Douglas *et al.* (1996a) reported that P450scc mRNA levels did not vary significantly over the course of mink gestation, suggesting its constitutive expression; therefore, the significance of the increase in P450scc mRNA observed in LH and $(Bu)_2$cAMP treated mink luteal cells is unclear.

Results of the current experiment revealed that the abundance of 3β-HSD, the enzyme responsible for the conversion of pregnenolone into progesterone, mRNA was stimulated by treatment of cultured mink luteal cells with FSH and $(Bu)_2$cAMP but not LH. Previous reports from our laboratory have shown that the abundance of 3β-HSD mRNA varies significantly over the course of mink gestation and has been correlated to progesterone production (Douglas *et al.*, 1996a). However, our findings argue for prolactin as the principal hormone in the regulation of the expression of this enzyme (Douglas *et al.*, 1996a; 1996c; and 1996d).

The enzyme P450scc is located on the inner mitochondrial membrane. The delivery of cholesterol from cellular stores and the outer mitochondrial membrane to the inner mitochondrial membrane is a regulated and rate limiting step in steroidogenesis (Crivello and Jefcoate, 1980; Jefcoate *et al.*, 1987). Several studies have proposed that the mitochondrial protein, StAR, may be involved in this cholesterol transport process (Clark *et al.*, 1994; Waterman, 1995; Lin *et al.*, 1995; Clark *et al.*, 1995; Sugawara *et al.*, 1995). In mouse MA-10 Leydig tumor cells and human granulosa cells, StAR mRNA was shown to be acutely regulated by tropic hormones and cAMP analogs (Clark *et al.*, 1995; Sugawara *et al.*, 1995). In the current experiment treatment of cultured mink luteal cells for 12 hours with 300 μM of $(Bu)_2$cAMP but not LH and FSH stimulated a 3 fold increase in the abundance of StAR mRNA. This response is comparable to the response of human granulosa cells which showed a 3-7 fold increase in StAR mRNA after treatment for 24 hours with 1.5 mM of 8-Br-cAMP (Sugawara *et al.*, 1995). It is also comparable to the response of MA-10 cells which showed that they were acutely responsive to $(Bu)_2$cAMP stimulation, since $(Bu)_2$cAMP increased StAR mRNA levels within 2 hours (Clarke *et al.*, 1995). Previous work in our laboratory has shown the StAR mRNA is present in the mink ovary during embryonic diapause, CL activation and early post-implantation but its levels do not vary significantly over the course of mink gestation.

In conclusion, treatment of cultured mink luteal cells with $(Bu)_2$cAMP, LH and FSH, stimulated progesterone production and mRNA for the steroidogenic enzymes P450scc and 3β-HSD. These results add further evidence that there is a luteotropic complex in this species and LH and/or FSH in addition to prolactin are needed for the normal function of the mink CL through gestation.

7.7 ACKNOWLEDGMENTS

We would like to thank Drs. T. Wise, F. Labrie and D. Stocco for the generous provision of cDNA probes for porcine P450scc, human 3β-HSD and mouse StAR. We would also like to thank Mr. M. Morrow for the care of the animals used in this experiment. Technical assistance was generously provided by Mrs. M. Dobias Goff. This project was supported by NSERC and MRC operating grants to BDM.

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7.9 STATEMENT OF PARTICIPATION

Experimental Design - Deborah Douglas, Bruce Murphy

Animal Handling - Deborah Douglas, Jianhua Song, G Moreau

Sample Collection - Deborah Douglas, Jianhua Song, G Moreau

Tissue Culture - Deborah Douglas

Slot Blot Analyses - Deborah Douglas

Progesterone Assay - G Moreau

Statistical Analysis - Deborah Douglas

Interpretation of Results - Deborah Douglas, Bruce Murphy

Paper - Deborah Douglas

8.0 GENERAL DISCUSSION

Prolactin is an essential hormone in the reactivation of the mink CL and is important for its regulation and function throughout gestation. The results of the experiments presented herein indicate that one mechanism by which prolactin exerts its luteotropic effects is through the regulation of luteotropic hormone receptors, namely the PRLr and LHr. In order to study the effects of prolactin on its own receptor, we cloned and sequenced a portion of the mink PRLr gene and monitored changes in the abundance of PRLr mRNA over the course of mink gestation. The mink PRLr displayed high nucleic and amino acid sequence homology with the PRLr sequences of a number of other species (Boutin *et al.*, 1989; Shirota *et al.*, 1990; Davis and Linzer, 1989; Boutin *et al.*, 1989; Scott *et al.*, 1992; Edery *et al.*, 1989). Four cysteine residues in the extra-cellular domain and two in the transmembrane region were conserved among all the species studied. In addition, two potential N-linked glycosylation sites were also conserved. This provides indirect evidence that these residues may be important for hormone binding, receptor structure and/or function.

Prolactin receptor mRNA and protein were present in the mink ovary throughout gestation. Levels were low during diapause but increased during CL reactivation and were highest during the early stages of post-implantation gestation. Prolactin receptor mRNA and protein levels closely paralleled changes in serum prolactin levels. Treatment of mink with the dopamine agonist bromocryptine, which prevents the pre-implantation rise in prolactin, also abolished the pre-implantation rise in prolactin receptor mRNA. In addition, prolactin treatment of anestrous mink, in which follicular development and ovulation were induced with exogenous hormones, stimulated a five fold increase in the abundance of PRLr over that of non-prolactin treated controls. Taken together, these data indicate that prolactin is involved in the regulation of its own receptor in the mink ovary. The ability of prolactin to up-regulate its own receptor has been demonstrated in a number of tissues and species. An inductive effect of prolactin was first postulated when it was observed that a renal capsular pituitary implant in hypophysectomized rats augmented hepatic PRLr approximately 48 hours after the increase in serum PRL (Posner *et al.*, 1975). In addition, prolactin secreting tumors were also found to be associated with elevated hepatic PRL receptor levels (Posner, 1976). Prolactin induction of its own receptor has also been demonstrated in rat lung (Amit *et al.*, 1985), liver (Amit *et al.*, 1985; Manni *et al.*, 1978; Barash *et al.*, 1988), and kidney (Barash *et al.*, 1986) as well as in the liver of the Snell-dwarf mouse (Knazek *et al.*, 1977) and the testes of the golden hamster (Klemcke *et al.*, 1984; Klemcke *et al.*, 1986). However, the results of the current experiments also revealed that *in vitro* treatment of cultured mink luteal cells with prolactin had no effect on the

abundance of PRLr mRNA. These discrepancies may indicate that extra-luteal factors in addition to prolactin may be involved in prolactin regulation of its receptors. Another possibility is that the culture conditions were not conductive to the up-regulation of prolactin receptor.

Luteinizing hormone is an important luteotropic hormone in most species, however, its role in the regulation of the mink CL is unclear. The results of the experiments presented here indicate that LH may play an important role in the reactivation and regulation of the mink CL. In order to determine the part LH playes in the reactivation of the mink CL, the transmembrane and cytoplasmic regions of the mink LHr gene was amplified by RT-PCR and sequenced. The amplified fragment was then used as a probe to determine changes in the abundance of LHr mRNA over the course of mink gestation. The mink LHr sequence had high nucleic and amino acid homology with the LHr of other species (McFarland *et al.*, 1989; Loosfelt *et al.*, 1989; Frazier *et al.*, 1990; Minegishi *et al.*, 1990; Gudermann *et al.*, 1992). Of 22 potential phosphorylation sites present in the intracellular region of the rat LHr (Segaloff and Ascoli, 1993), 19 were conserved in the mink sequence. The number and location of potential phosphorylation sites in the first and second intracellular loops were the same in the mink and rat. However, in the third intracellular loop one of the three potential phosphorylation sites in the rat was shifted from a threonine to a methionine in the mink. Hipkin *et al.* (1995) provided evidence which indicated that serine residues at amino acid positions 635, 639, 649 and 652 of the LHr were phosphate acceptors in response to hCG and PMA. In the mink, however, only two of these serine residues were conserved (639 and 652). In addition to the 19 potential phosphorylation sites conserved between the rat and mink, a further six sites were present in the cytoplasmic tail of the mink LHr. Segaloff and Ascoli (1993) reported that 20 cysteine residues were conserved among the four species they studied. Eleven of these were present in the transmembrane and cytoplasmic regions of the receptor. All cysteine residues were conserved in the deduced amino acid sequence of the mink LHr, including two cysteine residues at amino acid position 621 and 622 of the rat LHr, which are believed to be palmitoylated (Zhu *et al.*, 1995).

The abundance of LHr mRNA varied significantly over the pre-implantation and early post-implantation stages of mink gestation, in a manner which was strikingly different from that of the PRLr. LHr mRNA levels showed a transient increase at the time associated with CL reactivation. The levels then decreased but remained detectable through the rest of early post-implantation gestation. When the animals were treated with bromocryptine, the transient peak in LHr was abolished and LHr mRNA levels were reduced below that of the pre-treatment control. The abolition of the LHr mRNA peak by treatment with bromocryptine suggested that prolactin may play a significant role in the regulation of LHr

mRNA in this species. Results from the other experiments presented here support this conclusion. Prolactin treatment of animals, in which follicular development and ovulation were induced with exogenous hormones, showed an increase in the abundance of LHr mRNA over the non-prolactin treated controls. In addition, treatment of cultured mink luteal cells with prolactin resulted in a dose dependent increase in LHr mRNA. A role for prolactin in the regulation of LHr binding sites (Gibori and Richards, 1978; Jammes *et al.*, 1985) and/or mRNA (Gafvels *et al.*, 1992; Bjurulf *et al.*, 1994) has been suggested in a number of other species.

Changes in the abundance of LHr mRNA at the time of CL reactivation and the presence of LHr mRNA throughout the early post-implantation stages of gestation suggest a role for LH in the regulation of CL function in the mink. Further support for a role of LH in mink luteal function was demonstrated by the ability of LH and cAMP but not prolactin to stimulate increases in progesterone production in cultured mink luteal cells collected at various stages of mink gestation. These results are supported by a study by Murphy *et al.* (1993) in which they reported that over a 2 hour incubation, LH treatment induced increases in the production of progesterone by cultured mink luteal cells collected from mink during the post-implantation period of pregnancy. However, they are contradictory to a report by Stoufflet *et al.* (1989) in which they stated that LH had no significant effect on progesterone production regardless of the stage of gestation from which they were collected. The discrepancy in the response of luteal cells in these experiments may be due to differences in the doses of LH used and/or the culture conditions.

Although the results from the experiments presented here indicate a role for LH in the reactivation and maintenance of the mink CL, the mechanism by which LH exerts its luteotropic effects is unclear. Results from the mink luteal cell culture studies reported here showed that LH and cAMP were able to stimulate increases in the abundance of both P450scc and StAR mRNA. Therefore, it's possible that LH exerts its effects through one or both of these steroidogenic elements, especially since they are rate limiting steps in progesterone biosynthesis. It seems unlikely, however, since a reduction of LHr mRNA by treatment of pregnant mink with bromocryptine, and thus a reduction in the ability of LH to stimulate progesterone production, had no effect on the abundance of either P450scc or StAR mRNA in whole animal studies.

It is important to note that, although the results of the current experiments support a role for LH in the reactivation and maintenance of the mink CL, data from previous studies do not always support this view. For example, in a report by Murphy *et al.* (1981) it was shown that treatment of hypophysectomized mink with prolactin alone was able to reactivate the mink CL, stimulate progesterone production and induce implantation in some of the animals, while treatment of mink with LH had no effect on progesterone production.

A possible explanation for this observation is that the prolactin preparation used may have been contaminated with small amounts of LH, which allowed for the transient reactivation of the mink CL. In the case of the animals which were treated with LH alone, the animals may have been unable to respond to LH treatment because a prior exposure to prolactin is necessary to induce LH receptors. It is important to note, however, that in the same study it was found that treatment of hypophysectomized mink with prolactin and LH did not increase or extend progesterone secretion beyond that of prolactin treatment alone.

Taking all of the above information into consideration the following model was developed to explain the interactions of prolactin with luteotropic hormone receptors in the mink CL. Corpora lutea during embryonic diapause have very low levels of LHr but do contain significant levels of PRLr. Low levels of LHr are necessary so that the CL do not to respond to LH stimulation during the normal breeding season and become reactivated at an inappropriate time. This is important since diapausing CL are exposed to dramatic changes in LH levels as a result of continuing waves of follicular development, as well as mating induced LH surges. After the vernal equinox, as serum prolactin levels increase a concurrent increase in PRLr levels also occurs. This in turn increases the CL responsiveness to prolactin. Prolactin working through its cell surface receptor then induces an increase in LHr allowing the CL to become sensitive to the luteotropic effects of LH.

In addition to determining the effects of prolactin on luteotropic hormone receptors, its role in the regulation of progesterone biosynthesis was also examined. Specifically, the effects of prolactin on the abundance of P450scc, 3β-HSD and StAR mRNA were studied. There are two key rate limiting steps in progesterone biosynthesis. The first is the transport of cholesterol, the principal substrate for all steroidogenesis, across the mitochondrial membrane and the second is the conversion of cholesterol into pregnenolone. StAR has been proposed as the mitochondrial protein responsible for the movement of cholesterol across the mitochondrial membrane (Clark *et al.*, 1994), while P450scc is the enzyme responsible for the conversion of cholesterol to pregnenolone (reviewed by Miller, 1988; Hinshelwood *et al.*, 1993). Since prolactin is essential for the reactivation of the mink CL and progesterone production, it was hypothesized that it may exert its luteotropic effects at the level of these proteins. The results of the current experiments, however, indicated that the abundance of mRNA for these proteins did not change significantly during CL reactivation and early post-implantation gestation. In addition, prolactin was not found to have a significant effect on the abundance of mRNA for either of these proteins. In fact prolactin treatment of cultured mink luteal cells resulted in a decrease in the amount of StAR mRNA and treatment of anestrous mink in which follicular development and

ovulation were induced, with prolactin resulted in a decrease in the abundance of P450scc mRNA below that present in the non-prolactin treated controls.

The most profound effects of prolactin on progesterone biosynthesis were found in regards to its regulation of 3β-HSD mRNA in the mink CL. This enzyme is a key step in progesterone biosynthesis since it is responsible for the oxidation and isomeration of pregnenolone to progesterone (reviewed by Miller, 1988; Hinshelwood *et al.*, 1993). In the mink CL, changes in the abundance of 3β-HSD mRNA were found to be closely correlated to serum progesterone levels, since it was found that 3β-HSD mRNA levels were low during embryonic diapause but increased during CL reactivation and peaked during early post-implantation gestation. Treatment of animals with bromocryptine, to suppress their endogenous prolactin levels, prevented the pre-implantation rise in 3β-HSD mRNA levels. In anestrous animals, in which follicular development and ovulation were induced with exogenous hormones, subsequent treatment with prolactin was found to stimulate a transient increase in the abundance of 3β-HSD mRNA levels. In addition, treatment of cultured mink luteal cells also resulted in an increase in the abundance of 3β-HSD mRNA. Although these results indicate a key role for prolactin in the regulation of 3β-HSD mRNA in the mink CL, the significance of these findings is not clear, since 3β-HSD is not a rate limiting step in steroid biosynthesis. It is, however, possible that changes in the abundance of mRNA for this enzyme may reflect changes in the direction of steroidogenesis from the Δ5 to the Δ4 pathway with the resultant production of progesterone. This finding is consistent with the so called permissive role of prolactin on progesterone production ie. that prolactin does not directly stimulate progesterone but rather it maintains the biosynthetic machinery which allows other factors to induce progesterone production.

A permissive role for prolactin on progesterone biosynthesis is also supported by the fact that, in the experiments reported here, prolactin alone was unable to stimulate progesterone biosynthesis using two different models. In anestrous animals in which follicular development and ovulation were induced, prolactin treatment alone stimulated changes in mRNA levels similar to those seen during CL reactivation ie. there were increases in 3β-HSD, LHr and PRLr mRNA. However, in spite of these changes serum levels of progesterone remained below the sensitivity of the assay. In addition, treatment of cultured mink luteal cells with increasing concentrations of prolactin had no effect on the levels of progesterone in the culture media, while both cAMP and LH were able to stimulate modest increases. The inability of prolactin to stimulate cultured mink luteal cells to produce progesterone has been reported previously by Stoufflet *et al.* (1989). However in a report by Murphy *et al.* (1993) it was found that as little as 1 ng/ml of prolactin was able to stimulate a small (<11%) but significant increase in progesterone production.

In addition to the elements of the progesterone biosynthetic pathway which were discussed here, there are a number of other possible sites at which prolactin may be exerting its luteotropic effects. One important site may be at the availability of cholesterol as substrate for steroidogenesis. Cholesterol comes from three sources, *de novo* synthesis, intracellular cholesterol ester stores or through LDL (reviewed by Murphy and Silavin, 1989; Gore-Langton and Armstrong, 1988). Prolactin has been found to have significant effects on several elements involved in the regulation of intracellular cholesterol in other species. Numerous studies have shown that prolactin is able to stimulate ovarian CEH levels in the rat (Behrman *et al.*, 1970b; Behrmen *et al.*, 1971; Klemcke and Brinkley, 1980a; Klemcke and Brinkley, 1980b; Klemcke and Brinkley, 1980c). This is important since cholesterol released by the hydrolytic activity of CEH has been shown to subsequently serve as a major precursor for progesterone biosynthesis (Armstrong, 1968; Behrman *et al.*, 1970a; Behrman *et al.*, 1970b). Prolactin has also been shown to be involved in lipoprotein utilization. Prolactin enhances both HDL and LDL-induced progesterone accumulation by luteal cells from the pregnant pig (Rajkumar *et al.*, 1984), ferret (McKibbin *et al.*, 1984) and mink (Murphy *et al.*, 1984). If prolactin is indeed involved in the regulation of cholesterol availability in the mink CL its effects may have been masked in the current mink luteal cell culture experiments. This is due to the fact that 25-hydroxycholesterol was provided as substrate in these experiments. This form of cholesterol is able to pass directly through the cellular membrane and was used to eliminate the possibility that progesterone production by the cultured cells would be restricted by substrate available.

Another possible site for the luteotropic effects of prolactin in the mink is on the enzymatic degradation of progesterone. Several studies have shown that prolactin suppresses the enzyme 20α-hydroxysteroid dehydrogenase (20αHSD: Lamprecht *et al.*, 1969; Albarracin and Gibori, 1991; Albarracin *et al.*, 1994) in the rat CL. This enzyme is responsible for the conversion of progesterone to 20α-hydroxyprogesterone, a reduced steroid with weak to nonexistent progestational activity (Rennie and Davies, 1964; Wiest and Forbes, 1964; Talwalker *et al.*, 1966). However, it seems unlikely that alterations in the abundance of 20α-HSD constitutes a major effect of prolactin in the mink since the ratio of progesterone and 20α-hydroxyprogesterone appears to be relatively constant in the mink throughout gestation and 20α-hydroxyprogesterone concentrations make up less than 10% of the total progesterone in the mink (Murphy and Moger, 1977).

In conclusion, the results of the experiments presented here indicate that prolactin is an important regulator of luteotropic hormone receptors in the mink CL. The results revealed that prolactin was able to stimulate changes in the abundance of mRNA for both it own receptor and that of LH, using three different experimental models. Although, the results indicate that prolactin was necessary for the expression of mRNA for both these

receptors, their pattern of expression during embryonic diapause, CL reactivation and early post-implantation gestation were very different. In addition to its role in the regulation of luteotropic hormone receptors, the role of prolactin in the regulation of key steroidogenic elements was also examined. The results indicated that prolactin probably does not exert its luteotropic effects by altering the abundance of mRNA for either P450scc or StAR, which are two important rate limiting steps in progesterone biosynthesis. Prolactin does, however, appear to be important for the regulation of 3β-HSD, since prolactin was able to stimulate increases in the abundance of mRNA for this enzyme using three different experimental models. Inspite of the ability of prolactin to alter 3β-HSD mRNA abundance, the importance of this finding is not clear since 3β-HSD is not a rate limiting step in progesterone biosynthesis. It may, however, indicate a permissive role for prolactin in progesterone biosynthesis through a shift in the direction of steroidogenesis from the Δ 5 to the Δ 4 pathway. Finally, changes in the abundance of LH receptor mRNA during the pre-implantation gestation, as well as the ability of LH and cAMP but not prolactin to stimulate progesterone production in cultured mink luteal cells, indicate that LH as well as prolactin may be necessary for normal CL function in the mink.

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IMAGE EVALUATION
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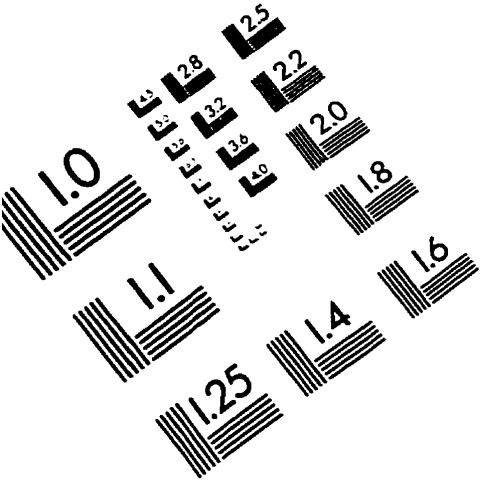

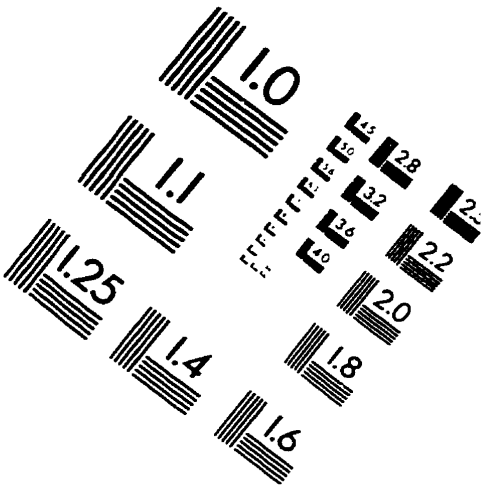

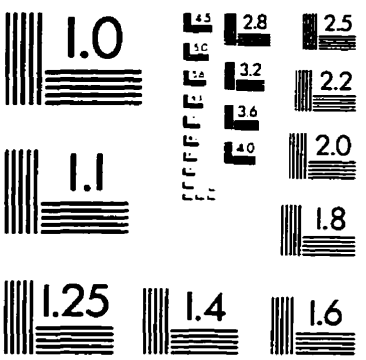

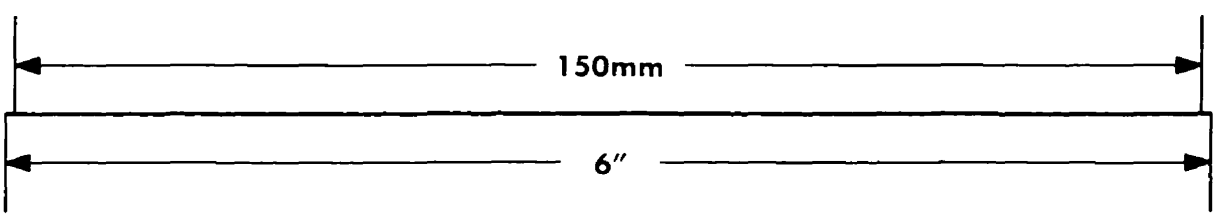

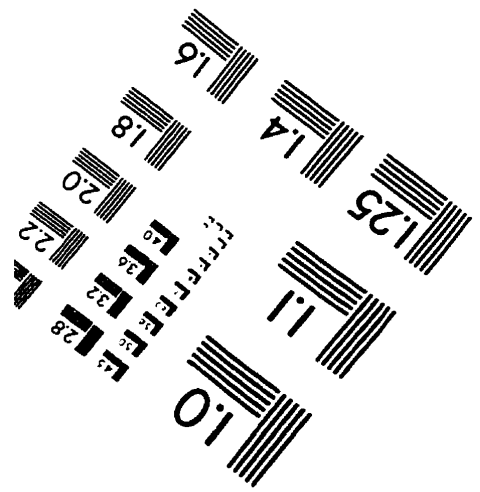

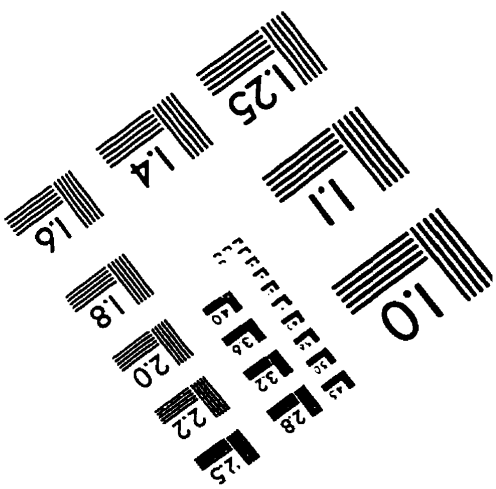

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