ABSTRACT

Title of dissertation: EFFECTS OF MODERATE CALORIE RESTRICTION ON OVARIAN FUNCTION AND DECLINE IN RHESUS MONKEYS

Julie Mei-Fen Wu, Doctor of Philosophy, 2006

Dissertation directed by: Professor Mary Ann Ottinger
Department of Animal and Avian Sciences

Calorie restriction (CR) has long been heralded as the only proven nutritional intervention for life extension. Recent data demonstrated that moderate CR also extended reproductive lifespan in female rats. The objectives of this project were to: 1) analyze general hormonal changes that occur with aging and menopause and 2) evaluate the effects (whether beneficial or detrimental) of moderate (30%) CR on ovarian function and decline in rhesus monkeys (*Macaca mulatta*).

Hormone analyses demonstrated elevated FSH and reduced INHB in Old monkeys, prior to menstrual cycle irregularity and alterations in E2 or P4. Our data are the first demonstration of this hormonal event occurring in monkeys. Furthermore, moderate CR did not impair normal ovarian function or aging.

Evaluation of three clinically available tests: day 3 FSH, the Clomiphene Citrate Challenge Test (CCCT) and the Exogenous FSH Ovarian Reserve Test (EFORT), demonstrated that CCCT is efficacious in monkeys, especially with the use of E2 and INHB. As such, CCCT is the most cost effective and best predictor of ovarian response. Responses were similar between CON and CR.

Oocytes from old short-term CON and CR monkeys were collected and fertilized with spermatozoa collected from normal males. Interestingly, CR appears to prolong ovarian responsiveness to exogenous gonadotropins and improve embryonic development *in vitro* in old female rhesus monkeys.

Microarray analysis of gene expression was conducted in luteinizing granulosa cells. A subset of responsive genes were identified that will require validation by via real time polymerase chain reaction (RT-PCR). These data will provide insight into potential mechanisms of direct action of CR on the ovary.

Therefore, the results of this study have provided evidence for the utility of the rhesus monkey as a model for human menopause. Additionally, moderate CR did not impair normal reproductive function or decline. We also confirmed the efficacy of the CCCT in rhesus monkeys and recommend its use as a diagnostic tool. Finally, CR improved ovarian response to exogenous gonadotropins and has beneficial effects on oocyte quality and subsequent embryo development.

EFFECTS OF MODERATE CALORIE RESTRICTION ON OVARIAN FUNCTION AND DECLINE IN RHESUS MONKEYS

by

Julie Mei-Fen Wu

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
2006

Advisory Committee:

Professor Mary Ann Ottinger, Chair
Professor Tom E. Porter
Professor William Hodos
Dr. Steven L. Monfort
Dr. Donald K. Ingram

To Roo, who first inspired me to study Animal Science.

ACKNOWLEDGMENTS

As most recent PhD graduates would likely agree, this thesis has been the proverbial labor of love for me. I began my doctoral degree not without a large degree of optimism and idealism. As time progressed, reality took its toll and I began to discern a feeling of inadequacy, as I watched my old college room-mates start their careers, and most enviably, start earning substantial paychecks! Fortunately, I was surrounded by people who influenced me in a positive way, and who deserve all the credit for guiding me and supporting me through this long and sometimes seemingly endless road.

I cannot begin these acknowledgments without first thanking my family. My mother, Ai-hsia Wu has always strongly advocated education and encouraged me to pursue whatever path I chose. My sister Frances Wu has been my friend, my cheerleader and occasional financier. She has done much for my self esteem, referring to me as Dr. Wu long before I ever deserved it. My father, Song Fong Wu has helped me through years of semi-dependency and never suggested there was another or better way. Finally, but not insignificantly, I must thank my husband Barry Adkins for being my strongest ally and for his ever constant belief in my abilities. At the end of the longest days, your warmth, affection and above all, humor kept me going.

I feel exceptionally fortunate to have worked with two wonderful advisors, Drs. Mary Ann Ottinger and Mary Zelinski, who both always consider people as a whole and who have taught me so much more than what appears in this thesis.

Mary Ann, I thank for her somewhat unconventional approach to advising and science. Her vision allowed me to pursue a project most students would not have the opportunity in which to partake. Mary is an exceptional role model both as a person and a scientist. Her eternal optimism and boundless patience are almost mind-boggling at times, but always something to aspire to myself.

I greatly appreciate the sound advice and recommendations of my Thesis Committee. Dr. Donald Ingram took a chance on a couple of bird folks and really made this all possible. Dr. Steven Monfort not only mentored me years before I began my degree, but also availed himself to me both professionally and personally whenever I needed it. Dr. William Hodos always contributed supportive and encouraging words in our meetings, and I appreciate his efforts in assisting me in my career search. I am also deeply grateful to Dr. Tom Porter for his assistance in preparing me for my comprehensive exams.

I must make a special note of thanks to two very special people. Nikki Thompson trained me when I first joined the group and always had plenty of food, chocolate and good conversation at the ready. Dr. Mahmoud Abdelnabi was like a father and mentor to me all rolled in one. He is the only person I know whose patience may rival Dr. Zelinski's.

To my wonderful friends who have been more like family to me than many of my close relatives. Jennifer Cui, who in the midst of raising two wonderful girls always made time for me as my confidante, sometimes therapist and always dear friend. Julie Hazelton, who can find humor in even the worst of situations; she one of the most intuitive, sensitive and generous people I know. Sultan

Jenkins, without whom I would have missed out on some of the best social events; his is always there for his friends and generous almost to a fault. Michael Quinn, I will always thing of as my eating and griping buddy. We had so much fun in that little office, people would come down the hall just to see what we were laughing about. Sara Pollack, my fellow animal lover and source for trivial information. She was happy to sing “Go Panda” whenever I needed it, even if it meant doing it over and over and over again. And last, but not least, thanks go to Brandon Sitzmann, for his sarcastic sense of humor and for encouraging me to say aloud, things I probably should just keep to myself!

Thank you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ONPRC	Oregon National Primate Research Center
OHSU	Oregon Health and Science University
NIA	National Institute on Aging
CR	calorie restriction
YCON	young control
YCR	young calorie restricted
OCON	old control
OCR	old calorie restricted
GnRH	gonadotropin releasing hormone
LH	luteinizing hormone
FSH	follicle-stimulating hormone
E2	estradiol
P4	progesterone
INHB	inhibin B
hCG	human chorionic gonadotropin
ELISA	enzyme-linked immunosorbant assay
RIA	radio immunoassay
BTB	break through bleeding
COS	controlled ovarian stimulation
ORT	ovarian reserve test
CCCT	clomiphene citrate challenge test
EFORT	exogenous follicle-stimulating hormone ovarian reserve test

CHAPTER 1

GENERAL INTRODUCTION

The average age of menopause in women is approximately 51 years, resulting in a post-reproductive period that extends for nearly one third of their lives (Treloar, 1981). Further, because many women have elected to delay reproduction, due to career and other considerations, they have encountered the reality of reproductive aging and ovarian aging. Consequently, the mechanisms involved in the processes of ovarian aging have gained increased visibility and relevance. The hypothalamic-pituitary-gonadal axis (HPG) is central to reproductive function (Figure 1.1). External cues, such as nutrition and stress can modify HPG function as well. In the ovary, as follicles mature, various hormones contribute to the different stages of recruitment and selection. The gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) have important roles in follicular development and ovulation and are regulated by the hypothalamus (via gonadotropin releasing hormone; GnRH), anterior pituitary gland and the ovary. In terms of the ovarian hormonal milieu, estradiol and progesterone are major players and have been well characterized. Additionally, the inhibins and activins have important roles in FSH regulation. Furthermore, there are a number of growth factors that have paracrine and autocrine effects as well.

The function of the HPG axis retains remarkable similarity across taxa. A period of reproductive immaturity is followed by a period of reproductive maturity

and activity, finally ending with a period (variable in length) of reproductive senescence and loss of function. Reproductive decline during aging is evident in nonhuman primates, domestic livestock, elephants, whales, and lions as just a few examples (Packer et al., 1998). Evolutionary hypotheses seek to answer the question of why menopause occurs; cellular hypotheses attempt to answer the question of how it occurs; and research models create opportunities to study these questions in the laboratory.

Historically, it has been believed that female vertebrates acquire 100% of their primordial follicles at or around the time of birth. This doctrine has recently been brought into question by Johnson and colleagues (Johnson et al., 2004) who demonstrated that ovarian follicular renewal may be possible in mice. Regardless of the process, however, by menopause the ovary is almost completely devoid of follicles. Throughout the reproductive lifespan of any female, the number of follicles that become atretic is much greater than the number of follicles that actually proceed through to ovulation. Thus, ovarian aging in one form or another is a consistent theme.

Chronological age is a useful, although not always accurate predictor of reproductive function and progression into peri-menopause. Although in women, the final menopause does not occur until mid-life, the initial changes leading to the climacteric occur years before, in their late 20's. Modern demands from education and career often delay childbearing to those same years, creating potential issues with fertility and pregnancy success. Therefore, useful diagnostics capable of evaluating ovarian function can save valuable time and

resources. Despite competing theories regarding the mechanism of the loss of follicles coincident with ovarian decline, ultimately reduced ovarian reserve is a consistent theme. Development of reliable methods with which researchers may predict the onset of, or assess the extent of ovarian decline would be extremely beneficial.

Furthermore, if menopause merely affected fertility, the study of ovarian aging would likely not be of such high priority. A number of other physiological systems are also affected by the sudden withdrawal of hormonal support associated with menopause, including: bone density, cardiovascular health, cognition and possibly some cancers (Johnson et al., 2004; Nappi et al., 1999; Prior, 1998; Sherwin, 2003). Given the wide range of physiological systems affected by menopause, the idea of delaying ovarian decline is attractive. To date, there are no proven methods of extending reproductive lifespan in primates, however, it is currently under study.

II. CURRENT HYPOTHESES ON OVARIAN AGING AND MENOPAUSE

Evolutionary Hypotheses and Theories

In humans, women experience a dramatic loss in ovarian function and subsequent menopause around the age of 50 (Treloar, 1981). Thus, with the average lifespan ~70-80 years, a woman will spend a significant portion of her life in a post-reproductive state. It has been suggested that menopause is simply a non-adaptive by-product of increased longevity in humans, since it seems contrary to maximizing Darwinian fitness (Blurton Jones et al., 2002). That is,

rapid advances in medicine, nutrition and health have allowed people to live much longer than they have in the past. One hundred years ago the average life expectancy was about ~30-40 years, so there was likely little to no post-reproductive life. Some scientists believe that not enough time has passed for an evolutionary effect; therefore, menopause is little more than a side effect of increased lifespan. Others have hypothesized that menopause evolved as "quality versus quantity" trade off.

Table 1.1 summarizes the following hypotheses and theories. Specifically, menopause may have evolved as a result of the rapid encephalization of humans in combination with acquisition of bipedal movement (Peccei, 2001; Williams, 1957). As humans became more adept at making and handling tools, brain size increased dramatically. Increased brain size resulted in a more altricial infant (helpless, with an extended parental care requirement). Additionally, evolution of bipedal locomotion resulted in pelvic alterations that made delivery more dangerous and difficult. It may be that as women aged, the mortality risk and energetic requirements of pregnancy, delivery and lactation became too great to risk. The extreme altriciality of human infants requires a large parental investment. Thus, the Good Mother Hypothesis (Alexander, 1974; Nesse and Williams, 1996; Sherman, 1998), states that there is a trade-off between increasing allelic contribution to the population (having more children) and ensuring the survival of present children. The Grandmother Hypothesis (Blurton Jones et al., 2002; Gibbons, 1997; Hawkes et al., 1998) is an extension of this theory, but it more directly addresses the post-menopausal period. It agrees with

the general risk of pregnancy at advanced ages, but also addresses the contribution of the grandmother to her grandchildren in assuring her own personal “fitness.” That is, a re-allocation of her reproductive effort to care for children already born, as opposed to production of offspring unlikely to survive maternal death. Although we usually think of this reproductive strategy in the context of humans and possibly non-human primates, elephants and pilot whales also show this type of social structure and have an extended period of post-reproductive lifespan. It is plausible that in some higher vertebrates, this strategy is useful in species that require extensive caretaking and energy investment during infant and maturing phases in the lifespan.

In a more general sense, the Disposable Soma Theory of Aging (Kirkwood, 2002) is based on the supposition that organisms favor reproductive systems over systems responsible for maintenance and repair. In other words, an animal will place the majority of its energy into reproductive development, and maintenance and aging occurs as a result of “neglected” systems. An example of this theory may be found in the reproductive history in some laboratory mice that exhibit a systematic decline in function over time. In the wild, however, the mouse’s reproductive ability may be perfectly functional up until its demise. The main difference of course is that in the laboratory, the mouse is in a protected environment and may live well beyond the typical age of a mouse in the wild. Thus, the Disposable Soma Theory states that the mouse has devoted most of its energy into maintaining reproductive function, but would need to continue only as long as the animal is likely to survive. Following cessation of reproduction, the

mouse deteriorates physiologically, as little energy has been delegated into maintenance of its somatic functions, ultimately resulting in many of the aging phenotypes observed in captivity. Finally, it is clear in some species, such as mice, that animals showing an age-related deterioration in their "fitness" are likely to be predated; whereas, animals in the laboratory are likely to survive much longer. This suggests that the aging phenotype observed in the laboratory would be rare in the wild, further supporting the validity of the Disposable Soma Theory. Like a protected captive animal, advances in human health and medicine have protected women and thus the rate of increase in lifespan may have exceeded the rate of increase in reproductive lifespan.

Cellular Hypotheses

Ovary-Driven Reproductive Aging

The depletion of follicular reserve and subsequent loss of fertility provide one explanation for how menopause occurs (vom Saal et al., 1994). It has historically been believed that the final population of oocytes in the adult female is established during a stage in embryogenesis where the primordial germs cells undergo a multitude of mitotic divisions and formation of oogonia (Wassarman, 1994). Cells enter meiotic prophase and remain arrested in the cell cycle as primary oocytes, until puberty and exposure to appropriate levels of gonadotropins. During the peri-pubertal phase, development of gonadotropin-dependent granulosa cells mediate oocyte growth. Depending on the species, some preantral follicles are recruited, and one or more antral follicle(s) are

selected for ovulation. At this point, the recruited oocyte awaits signals from the pituitary for ovulation and resumption of meiosis. Follicular cells in the recruited oocytes undergo functional changes resulting in production of progesterone and preparation for fertilization.

The prolonged cell cycle arrest just described is a unique feature of female vertebrate gonadal development. Thus, the follicular or ovarian reserve provides an exhaustible resource of oocytes and follicles that is established at or around the time of birth. Faddy et al. (Faddy et al., 1992) reported the age-related bi-exponential decline in follicles; however, the accuracy of those data are debatable. Arguments suggest the bi-exponential decline is an artifact of the log-linear transformation and that follicle depletion is in fact monophasic (Leidy et al., 1998). In any event, the ovarian follicular reserve declines during aging and the majority of follicles are lost in atretic processes (Faddy et al., 1992; Johnson, 2003). It becomes very interesting to examine not only the fundamental biology of this process, but also the potential for extending ovarian function with interventions known to affect overall lifespan.

Inhibin (INH) was first identified and characterized in 1932 and shown to be involved in the regulation of the pituitary gland. It is a dimeric, glycoprotein hormone consisting of an α subunit with either a β_A- or β_B- subunit, denoted as inhibin A (α -β_A; INHA) or inhibin B (α -β_B ; INHB; (Soules et al., 1998). Using *in situ* hybridization, Roberts et al. (Roberts et al., 1993) localized the expression of the inhibin subunits in human ovaries throughout the menstrual cycle. They determined that the α subunit was expressed in the granulosa cells of small

antral as well as in dominant follicles. Subsequent studies on circulating levels of inhibins throughout the menstrual cycles determined that INHB is the dominant inhibin produced in the early and mid-follicular phase, whereas INHA is the dominant inhibin synthesized in the late follicular and luteal phases (Groome et al., 1994; Groome et al., 1996).

Interest in INH has become more prevalent with respect to reproductive aging and menopause as its association with follicle stimulating hormone (FSH) has become more defined. Increased levels of FSH in older women are evident throughout the cycle; however, this elevation is most consistent in the early follicular phase. It remains unclear whether rising FSH levels are directly due to lowered levels of inhibins. Soules and colleagues (Soules et al., 1998) hypothesized that once the number of pre-antral follicles falls below some critical threshold, the subsequent drop in INHB may result in rising levels of FSH (Figure 1). Welt and colleagues (Welt et al., 1999) compared daily menstrual cycle hormone levels in younger and older cycling women to characterize the relationship between the inhibins and the menopause-associated rise in FSH. They determined that INHB remained lower among older cycling women throughout the menstrual cycle, while estradiol-17β (E_2) and FSH in the older women varied (either higher or lower than the young at specific times) as compared to FSH levels in younger cycling women. Therefore, FSH did not appear to be consistently predictive of an individual's reproductive system status. Overall, these data confirmed an inverse relationship between INHB and FSH as well as a general decline in INHB, INHA and progesterone (P4) prior to

detectable differences in circulating Estradiol levels. The decrease in INHB appears to be one of the earliest hormonal events that may lead to the age-associated increase in FSH (Klein et al., 2004; Welt et al., 1999). This lends credence to the hypothesis that reduced follicle numbers and consequently reduced INHB levels, lead to the monotropic rise in FSH observed in peri-menopausal women.

Increased levels of FSH during reproductive aging have been documented across several species. de Souza et al. (de Souza et al., 1998) demonstrated that endocrine changes occur with ovarian aging in sheep and that the pattern of change was similar to that observed in women. Specifically, there were significantly fewer antral follicles available for development in older ewes during the early luteal phase, despite similar ovulation rates compared to their younger counterparts. Consequently, INHA was reduced in both the follicular and luteal phases in older animals, and this was associated with increased concentrations of FSH during the luteal phase. Estradiol levels, however, were similar between the two age groups. These data are consistent with the view that there is an overall depletion of the follicular pool during the process of aging. The maintenance of similar number of ovulations in older animals suggests that a greater proportion of the follicular pool in an aging female is promoted through to ovulation. At some point, there appears to be insufficient number of developing follicles to produce the hormonal support necessary to stimulate hypothalamic GnRH and the subsequent preovulatory LH surge. This diminished

responsiveness and coincident decline in ovarian function snowball, leading to eventual reproductive failure.

Brain-Driven Reproductive Aging

There is evidence suggesting that the age-related increase in FSH levels ultimately leads to an accelerated follicular loss and subsequent ovarian failure. Changes that occur in the temporal pattern and synchrony of neurochemical and neuroendocrine signals may trigger the cascade of peri-menopausal events. It has been suggested that miscommunication between the brain and the pituitary-gonadal axis occurs as a result of dampening and desynchronization of neural signals (te Velde et al., 1998).

Meredith et al. (Meredith et al., 1992) researched the effect of unilateral ovariectomy (ULO) on the rate of loss of primordial follicles. Young and middle-aged rats were ovariectomized unilaterally and observed for changes in the loss of primordial follicles. The investigators determined that ULO triggered an increased loss of follicles, but only in the old rats. A subsequent study by Anzalone and colleagues (Anzalone et al., 2001) showed that ULO reduced follicular reserve in young and old rats. The effects of ULO were then compared in young virgin and middle-aged (MA) breeder female rats relative to sham-operated controls. Results revealed that ULO reduced ovarian follicular reserve to levels similar to MA control rats. Although preovulatory estradiol-17β (E_2) levels were found to be similar between groups, there was reduced follicular reserve and significantly lower amplitude in the LH surge on the evening of

proestrous. Moreover, the reduction in the LH surge was correlated with numbers of resting follicles. Interestingly, despite similar follicular reserves in the young ULO and MA control rats, MA rats still showed lower peak LH levels as well as fewer regular cycles. Young ULO animals also had increased FSH levels on the morning of estrous (likely a compensatory response), while MA ULO rats did not. This elevated FSH release in young ULO rats was associated with an increase in follicular development so that the number of preovulatory follicles in the single ovary of these animals was similar to the number of preovulatory follicles in both ovaries of the young controls. Therefore, experimental reduction in ovarian reserve affected the LH surge, ovulation and cyclicity in both young and MA ULO animals. However, young ULO females demonstrated a compensatory response by raising FSH levels despite reduced follicular reserve, suggesting a fundamental age-related alteration in the regulation of FSH unrelated to follicular reserve.

Limited data are available suggesting an age-related alteration in the central LH surge mechanism in peri-menopausal women (Park et al., 2000). Estradiol challenge in young and peri-menopausal women showed the predicted transient decrease in LH and FSH levels, presumably associated with negative feedback at the level of the hypothalamus. Following this initial decrease, however, seven out of nine of the young participants exhibited a LH surge, while a similar surge occurred in only one of the eight peri-menopausal women. Therefore, it appears that the peri-menopausal HPG axis may have changes that involve more than sub-normal ovarian function.

Isolating age-related changes in hypothalamic response from ovarian aging is difficult; however, a number of studies have been conducted using the rodent model. These studies have examined the response of the HPG axis at various stages in the life cycle of the female as well as the functional changes of the hypothalamus and pituitary gland with aging. Wise and colleagues (Wise, 1991) have reported decreased amplitude and frequency in LH secretion in older rats and a decline in the number of activated GnRH neurons, despite normal cycles (Lloyd et al., 1994; Scarbrough and Wise, 1990). These papers by Wise and colleagues and many other papers point to alterations in the systems that modulate GnRH as key elements of the reproductive decline. Overall, these studies indicate that hypothalamic response decreases, potentially in tandem with declining ovarian function (Micevych et al., 2003; Mills et al., 2002; Wise, 1991).

MODELS OF OVARIAN AGING AND MENOPAUSE

Finding appropriate animal models for studying menopause is a debated subject with many aspects. The clinical definition of menopause is the cessation of spontaneous menstrual cycling for at least one year. Primates are unique in the animal kingdom with regard to the extensive menstrual sloughing of the endometrial lining. Thus, in the strictest sense of the word, menopause research is only possible in animals that experience menses. Human and even nonhuman primate research can be prohibitive due to cost, complex variables in lifestyle, history, and time required to study aging systems. Therefore, there is a great

need for alternative models of menopause. The ideal menopause model would have similarity to endocrine and neuroendocrine aspects to human ovarian biology. Additionally, if possible, the model should have reproductive traits which can be manipulated in the laboratory, i.e. transgene or natural mutations resulting in accelerated or delayed example of ovarian aging. Therefore, it is important to be able to compare normal aging to accelerated and/or delayed models as a way of elucidating the process as well as the underlying mechanisms contributing to the process of aging. This section will present some of the current research on models that have been developed and are being used to study menopause and ovarian aging (Table 1.2).

Rodent Models

Rodent Ovarian Biology. Most rats will become reproductively mature at approximately 5 weeks of age. At reproductive maturity, they have an estrous cycle that lasts 4-5 days regardless of seasonal changes (vom Saal et al., 1994). Both rats and mice will begin to exhibit periods of persistent estrous, which is associated with elevated, constant levels of estradiol, low levels of progesterone, and a lack of LH surges and ovulations (vom Saal et al., 1994). The tonic levels of estradiol lead to stimulation and cornification of the vaginal epithelium, resulting in a state of persistent vaginal cornification. Laboratory mouse and rat strains differ with regard to age of ovarian decline both within and between species and may occur from 6 to 18 months of age, depending on strain (Felicio et al., 1984). Ultimately, mice and rats will enter a final stage characterized by

low plasma estradiol and progesterone levels, as well as little to no remaining developing ovarian follicles (Lu et al., 1979).

The use of rodent models in ovarian aging research is manifold. They are relatively short-lived and the availability of homogenous laboratory strains permit controlled research experiments. As such, ovarian function in rodents is well characterized. Furthermore, genetic manipulation of specific genes has made it possible to study aspects of ovarian decline in transgenic models as well.

Bax$^{-/-}$ *Mouse.* Delayed ovarian aging has been documented in a knock-out mouse model: the *Bax*$^{-/-}$ mouse. The exact mechanism by which ovarian reserve depletion occurs is unknown, although, atresia and apoptosis are certainly involved. Bax is a member of the Bcl-2 family of proteins which are considered to be pivotal in the regulation of cell death pathways (Kim, 2005). The Bcl-2 family members are generally classified as either pro-apoptotic, such as Bax, Bid and Bad; or anti-apoptotic, such as Bcl-2 and Bcl-X_L (Kim, 2005). These proteins are believed to exert their apoptotic effects via hetero- and homo-dimerization (Liu et al., 1996; Sedlak et al., 1995; Yang et al., 1995) and the ability to form membrane channels, thereby influencing ion or protein transport (Garcia-Saez et al., 2005; Tilly et al., 1995).

Bax has been localized in both granulosa cells and oocytes (Jurisicova et al., 1998; Kugu et al., 1998; Perez et al., 1999). Upon gross examinations, 20-22 months aged *Bax*-deficient female mice exhibited uterine hypertrophy as compared to their aged matched controls (Hoyer et al., 2001). Morphological

analyses revealed the presence of multiple follicles at varying stages of development, including large antral follicles with visible oocytes. No indication of ovulation (presence of corpora lutea) was noted; however retrieval of oocytes following a superovulation protocol indicated that a mixture of normal, mature and abnormal oocytes were present in these animals. As expected the aged matched control ovaries chiefly consisted of stromal tissue, lacking evidence of follicles or oocytes.

Investigators performed morphometric analyses to determine if the sustained follicle endowment observed in *Bax*-deficient female mice was attributable to a greater initial ovarian follicle reserve (Hoyer et al., 2001). Neonatal wild-type and *Bax*$^{-/-}$ mice were found to have similar numbers of non-atretic primordial and primary follicles. Shortly after puberty, however, *Bax*$^{-/-}$ mice exhibited three times the ovarian reserve of their wild type counterparts. The authors hypothesized that the *Bax* deficiency may have granted some protection to the granulosa cells and oocytes against apoptosis. This model provides a unique perspective by delaying, if not eliminating ovarian senescence in the mouse. *Bax* levels have been shown to be elevated with the initiation of cell death in the human ovary (Jurisicova et al., 1998). Thus, the *Bax*-deficient female mouse model is a useful and intriguing model for studying menopause and the decline in the ovarian follicular reserve in women.

VCD-Treated Rodent. Rodents treated with 4-Vinylcyclohexene diepoxide (VCD) have been shown to exhibit accelerated ovarian failure. VCD is an

industrial chemical that is produced during the production and manufacture of insecticides, plasticizers, antioxidants, flame retardants and rubber tires (Doerr et al., 1995). It is being studied with regard to its ovotoxic effects and risk factor for premature menopause in women; however it also provides an interesting model for accelerated ovarian aging (Doerr et al., 1995; Springer et al., 1996). VCD will selectively destroy primordial and primary follicles when administered repeatedly to mice and rats (Doerr et al., 1995; Springer et al., 1996). The exact mechanisms by which the ovotoxicant VCD acts to initiate atresia are still largely unknown; however, it is believed to act by accelerating the natural processes of atresia (Borman et al., 1999; Hu et al., 2001). Induction of premature ovarian failure is thus possible via depletion of the pool of primordial follicles. The mechanism of action by which VCD acts is thought to be via pro-apoptotic pathways (Mayer et al., 2002).

After 30 days of treatments (80 mg/kg VCD per day), Fischer-344 rats had significantly reduced numbers of preantral follicles (Hooser et al., 1994). There were no apparent ultrastructural difference between groups; however, circulating levels of FSH were elevated by 120 days in VCD-treated as compared to vehicle animals. Furthermore, cyclicity was disrupted in VCD-treated rats by 360 days (Hooser et al., 1994). Experiments in mice yielded similar results: VCD-treated mice exhibited elevated levels of circulating FSH and reduced estradiol, at an age where control hormone levels were still normal (Mayer et al., 2004). Secondly, androgen levels in VCD-treated mice have also been shown to be elevated, similar to post-menopausal hyperandrogenic women (Mayer et al.,

2004). Combined, these data demonstrate that the accelerated time-frame of onset of ovarian senescence in the VCD-treated mouse supports its use as a menopause model, particularly of premature menopause. Moreover, this model may provide insight into the apoptosis-regulated pathways that likely lead to ovarian depletion and consequently menopause in women, whether normal or premature.

Foxo3a$^{-/-}$ Mouse. The *Foxo3a$^{-/-}$* mouse is another model for accelerated ovarian aging. Foxo3a is a member of the mammalian Foxo subfamily of forkhead transcription factors, including Foxo1, Foxo4 and Foxo6 (Brenkman and Burgering, 2003). Foxo transcription factors may be considered analogous to the DAF-16 transcription factor (and thus part of the DAF-2 pathway) in the roundworm (*C.elegans*; Kenyon et al., 1993). This is of considerable interest given exciting research which has shown that loss-of-function mutations in DAF-2 extends lifespan in the roundworm (Hosaka et al., 2004; Kenyon, 2001). The role of Foxo genes in mice is currently being explored; however it is clear that the individual genes (foxo1, foxo3a and foxo4) are functionally diverse (Burgering and Medema, 2003). *In vitro* data suggests a role for Foxo genes in cell cycle arrest, apoptosis and specific stress responses (Castrillon et al., 2003; Tran et al., 2003).

More specifically, Foxo3a has been found to be an essential regulator and suppressor of follicular activation (Castrillon et al., 2003). Investigators (Castrillon et al., 2003) generated a *Foxo3a$^{-/-}$* mouse bearing a null mutation in

the *Foxo3a* locus. These mice appeared outwardly normal up to 48 weeks of age, with no differences in body weight, or increases in cancer, or mortality. With regard to reproduction, however, Castrillon et al. reported sterility in these mice by 15 weeks of ages, despite normal sexual maturation (based on first litter). Histological analyses of ovaries indicated normal ovaries at postnatal day 3 (PD3), but by PD8, *Foxo3a*$^{-/-}$ ovaries were consistently enlarged. These mice exhibited early follicular activation, maturation and atresia, consequently resulting in early depletion of ovarian reserve. By 20 weeks, *Foxo3a*$^{-/-}$ females demonstrated classic signs of hypogonadotropic (elevated FSH and LH), hypogonadism, typical of premature ovarian failure. The authors suggest that these results indicate a role for Foxo3a specifically in follicular growth, but not other aspects of follicular maturation and reproduction. Thus, these data suggest that accelerated follicular initiation may be an underlying cause for premature ovarian failure.

FSH-R $^{+/-}$ *Mouse.* As its name implies, follicle-stimulating hormone (FSH) is the main hormone responsible for the processes involved in folliculogenesis, including follicular growth, and differentiation (Hillier, 2001). FSH receptors are found exclusively on the granulosa cells in the ovary, and the Sertoli cells in the testis; therefore all of its actions occur in these tissues (Camp et al., 1991; Zeleznik et al., 1974). FSH is essential for follicular maturation and the synthesis of estradiol from the granulosa cells via theca-derived androgen aromatization (Hillier, 2001). In addition, rats treated with FSH (10 μg/injection, twice daily)

have showed a decrease in DNA fragmentation and apoptosis in the ovary, suggesting a protective effect of the gonadotropin (Billig et al., 1994). Furthermore, some investigators have proposed the idea that ovarian follicles in aging females may become refractory to gonadotropin stimulation, impairing ovarian response and function (Gosden et al., 1983). These data, along with previously cited data reporting the elevated FSH levels coincident with ovarian decline, underline the need to elucidate the role of the FSH receptor in age-related ovarian failure.

The follitropin receptor knockout (FORKO) mouse was first characterized by Dierich and colleagues (Dierich et al., 1998). Female FORKO (FSH-$R^{-/-}$) mice are infertile and their ovaries are significantly smaller than their wild-type littermates due to a lack of large Graafian follicles and absence of corpora lutea (Danilovich et al., 2002). Conversely, FSH-R haplo-insufficient mice did reach reproductive maturity, and it occurred earlier than in their wild-type counterparts. Litter sizes in the FSH-$R^{+/-}$ mice were also lower at all observed ages (3, 7 and 12 months). Gross examination of ovarian histology in 3-month old mice showed little differences between wild-type and FSH-$R^{+/-}$; however, closer inspection revealed evidence of increased numbers of atretic follicles in the FSH-$R^{+/-}$ animals. By 7 months, there was an increase in the numbers of abnormal looking follicles, with irregular-looking or double oocytes. Additionally, although there was no difference in the total number of follicles in 3-month FSH-$R^{+/-}$ versus wild types, 7 month FSH-$R^{+/-}$ ovaries had significantly reduced numbers of oocytes as compared to their age-matched controls. The accelerated loss of

oocytes may be attributable to increase cell death, as the percentage of atretic follicles was much increased in the FSH-$R^{+/-}$ animals, at all ages (Danilovich and Sairam, 2002).

Danilovich et al., (Danilovich et al., 2003) have also reported on the endocrine alterations in the FSH-$R^{+/-}$ haplo-insufficient mouse. Young FSH-$R^{+/-}$ mice (3 months) have reduced estradiol, but similar pituitary gonadotropin levels to their wild-type counterparts. By 7 months, however, FSH-$R^{+/-}$ mice exhibit elevated gonadotropins and reduced estradiol levels (coincident with reduced numbers of ovarian follicles). These data indicate that the FSH-R +/- mouse experiences accelerated ovarian aging and may be a useful model for studying menopause (Danilovich et al., 2003). Furthermore, the authors suggest that these studies support the hypothesis that declining FSH-R and increasing FSH levels in aging females is a result of increased ovarian resistance to follicular development (Danilovich et al., 2003).

Primate Models

Human Ovarian Biology

In the U.S. menarche occurs in young women at an average age of 12.5 years (Abma et al., 1997). Menstruation is unique to primates and occurs as a result of sloughing of the endometrial lining. The menstrual cycle consists of the follicular phase and the luteal phase, divided by ovulation. Each phase lasts approximately 14 days, resulting in an median menstrual cycle length of 28 days (Vollman, 1977). Clinical menopause is defined as the period of a woman's life

one year after the cessation of menstrual cycles occurring at a median age of 51 years (Treloar, 1981). The events leading to the climacteric begin years earlier and are termed the peri-menopausal transition. Data collected by the Center for Disease Control (CDC) have shown that there is a significant age-related decrease in the incidence of pregnancy and age-related increases in spontaneous miscarriage, ectopic pregnancies and chromosomal abnormalities in young women (<30 years; (Ventura et al., 2004).

The monotropic rise in follicle-stimulating hormone (FSH) is the hallmark event indicating the onset of reproductive decline in women, showing significant increases in women prior to menstrual irregularity or other endocrine changes (Akande et al., 2004). Increased levels of FSH are evident throughout the cycle; however, the difference is most consistent in the early follicular phase. Day 2-5 FSH levels have been shown to correlate well with ovarian reserve and provide a commonly used biomarker in clinical practices to evaluate and predict pregnancy success via artificial reproductive technologies (Navot et al., 1994).

Use of human subjects for investigating reproductive senescence permits the most direct application of research into practice. Fortunately, the increased demand for human artificial reproductive technology (ART) has supported the rapid advancement of research in human reproduction. Unfortunately, some of this demand may be due to the trend in postponement of childbearing and the increased incidence of infertility with age (te Velde et al., 1998). Aging in the ovary appears to play a more important role in declining fertility than uterine aging (Blurton Jones et al., 2002). Navot and others (Navot et al., 1994)

demonstrated that when age of the oocyte donor was controlled, there were no significant differences in the delivery rate between young and old recipients. Within older cohorts, however, there may be differential fertility; thus, ovarian age is not necessarily dictated by chronological age (Bukman and Heineman, 2001). Furthermore, ovarian age may be determined by ovarian reserve, and this is dependent on the pool of remaining follicles as well as the quality of the oocytes.

DNA microarray analayses of luteinizing granulosa cells by Chin et al. (Chin et al., 2002) demonstrated differences in gene expression between women with normal or decreased ovarian reserve. Diminished ovarian reserve was determined based on day 3 FSH and peak serum E_2 levels and number of oocytes retrieved following an ovarian stimulation protocol. The authors admit the difficulty in interpreting these data, given small sample sizes, differential responses within groups, and inconsistent gonadotropin stimulation of subjects. It is still of great interest, however, to note that there were changes in a few specific genes. Although inconclusive, these data provide a basis for identifying specific gene targets that vary within the ovary during aging that will be useful for future research on ovarian aging and menopause in women.

Rhesus Monkey Ovarian Biology

Since the early 1900's, the rhesus monkey *(Macaca mulatta)* has been considered a useful model for reproductive studies in women (Heape, 1900). Female rhesus monkeys are pubertal by 2.5 to 3.5 years of age and exhibit menstrual cycles approximately 28 days in length, similar to women. Hormonal

and menstrual similarities to women have made the rhesus monkey a favored model in which to research ovarian function. Furthermore, rhesus monkeys experience a reproductive decline much like that of human menopause at approximately 24 years (Gilardi et al., 1997). One major difference in the menstrual cycle of humans versus rhesus monkeys however, is the existence of a breeding season in the latter. Both indoor- and outdoor-housed rhesus monkeys experience a breeding season that varies between primate facilities, but generally runs from September through May. Thus, further characterization of the onset of ovarian decline in rhesus monkeys was necessary to validate it utility as a model for human menopause.

The first report of a longitudinal assessment of menstrual patterns in aging rhesus monkeys was performed by Gilardi, et al. (Gilardi et al., 1997). Gilardi, et al. (Gilardi et al., 1997) reported urinary hormone profiles for 26 peri-menopausal rhesus monkeys. Menstrual records were documented for 12 months prior to the urine collection period. Subsequently, daily urinary estrone conjugate (E_1C) and pregnanediol-3-glucuronide (PdG) were analyzed for 12 weeks in female rhesus monkeys aged 20.5 to 29.5 years (average 23.5). As expected, the younger monkeys (less than 25 years) menstruated regularly, while the older monkeys demonstrated an increasing menstrual irregularity. Similar to women who experience low estradiol and irregular progesterone profiles with the onset menopause, menopausal rhesus monkeys had low E_1C levels as well as irregular patterns of PdG. These data initially suggested parallel events in rhesus monkeys and women with regard to menopausal onset.

Further research by this group analyzed urinary FSHβ as well as circulating inhibin B (INHB) in rhesus monkeys (Shideler et al., 2001). As discussed earlier, elevated circulating FSH is the hallmark event associated with the onset of ovarian decline in women. Twenty female rhesus monkeys between 18-26 years were analyzed for menstrual cycling, circulating INHB (2 samples collected between menstrual cycle day 3-5 and day 10-12), as well as daily urinary E_1C, PdG and FSHβ for one year. In agreement with the previous study, irregular menstrual cycling was associated with reduced urinary E_1C levels. Additionally, Shideler, et al. (Shideler et al., 2001) showed that urinary FSH levels were elevated in aged females. Unlike women, however, this elevation was only detectable after age-related menstrual irregularities were observed. Furthermore, decreases in circulating INHB levels were only detectable in ovariectomized monkeys as compared to normally cycling females. Therefore, the researchers acknowledge some key differences between the onset of menopause in rhesus monkeys and women.

Other Nonhuman Primates

Very little data exist characterizing menopause in other nonhuman primate species (Bellino and Wise, 2003). Menopause is simply the cessation of menstrual cycling; thus, by definition this is a state that may be achieved by any animal that experiences periodic endometrial sloughing and vaginal bleeding, including baboons, chimpanzees, gorillas and orangutans (Collins et al., 1975; Dahl et al., 1987; Gould et al., 1981). The baboon has been considered a useful

model for various reproductive studies, due to their similarities in menstrual cycle characteristics (Kraemer et al., 1977). Furthermore, the swelling of the perineal sex skin permits the noninvasive detection of the follicular phase and approximate time of ovulation (Domb and Pagel, 2001; Kraemer et al., 1977). Despite the use of the baboon in pregnancy and endometriosis research, however, very little had been reported on baboon menopause and the onset of reproductive decline. Menopause research in the great apes has been even less prevalent in the literature.

EVALUATING MENOPAUSE: OVARIAN RESERVE TESTS

Considering the high price of assisted reproduction, it is often considered prudent to estimate the probability of success prior to costly hyperstimulation and *in vitro* fertilization (IVF) regimes. Age of menopause in women may be highly variable, ranging for 10-15 years. Common to all pre- and peri-menopausal patients, however, is the monotropic rise in FSH, which may be attributed to changes occurring within hypothalamus and/or reduced feedback from the ovary itself. Evidence for hypothalamic changes with age have been suggested using artificially reduced follicle numbers via unilateral ovariectomy in rats (Anzalone et al., 2001). Conversely, comparisons between young and peri-menopausal women have showed reduced levels of inhibin B (INHB), elevated activin with no change in follistatin, giving support to an ovarian role in the rising levels of FSH in menopausal patients (Reame et al., 1998). Of note is the fact that many of these

hormonal changes occur prior to any measurable change in estradiol, progesterone or luteinizing hormone (Klein et al., 1996a).

d3 FSH. Ovarian reserve may be assessed clinically, by early follicular phase FSH level. Serum FSH concentration is commonly used as a predictor of fertility and likelihood of pregnancy. Early follicular phase FSH (day 2-5) has been shown to correlate well with ovarian reserve. Measurement of basal FSH alone, while extremely useful, can be somewhat variable. Thus, a number of tests have been developed that when used in conjunction with basal FSH levels, have been successful in predicting ovarian response. In general, most of these tests rely on assessing hormonal response to ovarian stimulation, whether at the level of the hypothalamus, pituitary or direct action on the ovary.

EFORT. Fanchin and colleagues (Fanchin et al., 1994) recently developed a supplemental protocol to the standard FSH measurements to assess ovarian reserve. The exogenous follicle stimulating hormone ovarian reserve test (EFORT) significantly improved the predictive value of basal FSH alone for IVF response. Subjects were injected with purified FSH on cycle day 3; blood was drawn prior to and 24 hours after FSH administration. Concomitant analysis of basal FSH and change in E2 following treatment was found to be effective in differentiating between good and poor responders to IVF. Change in INHB following EFORT, has also been shown to correlate well with response to IVF (Dzik et al., 2000). Women with both low baseline INHB levels as well as reduced INHB response to EFORT were less responsive to IVF treatment.

CCCT. The clomiphene citrate challenge test (CCCT) is a similar and also highly effective test of ovarian reserve. CCCT has been proven to be 93% effective in predicting infertility and was more successful in identifying low ovarian reserve patients than basal FSH alone (Kahraman et al., 1997). In young, healthy ovaries, ovarian reserve may be associated with an ample number of developing follicles. Administration of clomiphene citrate (CC), a hypothalamic estradiol receptor blocker, results in upregulation of FSH and LH from the pituitary.

Clomiphene citrate is an orally active non-steroidal chemical that is a mixture of two isomers: zuclomiphene and euclomphene; zuclomiphene being more active of the two (Speroff et al., 1999). Clomiphene itself, is weakly estrogenic, however due to its ability to bind receptor for long periods of time, it reduces negative feedback and increased gonadotropins, likely via activation of GnRH secretion. This stimulates follicular growth and subsequent production of inhibin and estradiol (Speroff et al., 1999). In the case of a peri-menopausal ovary, reduced follicle number results in a reduced inhibin response and presumably reduced negative feedback at the level of the pituitary and/or hypothalamus.

In human clinical trials, the CCCT is commonly used in conjunction with day 3 (with onset of mense as day 1 of the menstrual cycle) FSH levels to predict relative ovarian reserve. Patients are hormonally monitored to determine cyclicity, using P4 and LH. Basal FSH and E2 are measured in early follicular phase (days 2-5), and 100 mg clomiphene citrate is administered orally once

daily in mid follicular phase (days 5-9). FSH and E2 are measured in late follicular phase (days 9-11). The results of the basal versus clomiphene citrate-stimulated FSH and E2 may be used to predict relative IVF success. Cessation of CC results in suppression of FSH, likely via endogenous ovarian inhibins. INHB may be measured in combination with the CCCT as a direct measure of ovarian responsiveness. It has been shown that women with normal ovarian reserve have higher granulosa cell INHB production as compared with women with diminished ovarian reserve (Hofmann et al., 1998). Thus, the results of the CCCT are evaluated considering that younger women with a large ovarian reserve will respond to CCCT with a dramatic change in estradiol, comparing basal to stimulated hormone levels. Older women with significantly reduced ovarian reserve respond to CCCT with little to no change in estradiol, when comparing basal to stimulated hormone levels.

Ovarian Volume. Ultrasound may also be performed to evaluate ovarian activity in response to gonadotropin stimulation. Decreased ovarian volume may be observed earlier than initial rising concentrations of FSH (Syrop et al., 1995). Patients with ovaries less than 3 cm^3 were associated with a poorer response to human menopausal gonadotrophin stimulation than similarly aged patients with larger ovaries (Lass et al., 1997). Lass and Brinsden (Lass and Brinsden, 1999) reviewed the utility of ovarian volume in reproductive medicine and cited evidence for a correlation between ovarian volume and the number of primordial follicles in older women (>35 years). Additionally, ultrasound may be used to assess the number of small, antral follicles that has been shown to correlate

negatively with age (Scheffer et al., 1998). This is a relatively non-invasive and inexpensive method by which to evaluate ovarian reserve that may be used with other hormonal parameters.

DELAYING MENOPAUSE AND OVARIAN AGING

Calorie Restriction and General Aging

Calorie restriction (CR) is the only non-genetic method of altering longevity and other biological processes associated with aging. To address the relevance of this nutritional intervention to human aging, the National Institute on Aging began the first study of CR and aging in non-human primates using rhesus monkeys to determine its potential application in humans. These animals were gradually restricted until a 30% calorie restricted diet had been reached. The diet was supplemented with additional vitamins and minerals to guard against malnutrition (Ingram et al., 1990). Several endpoints have been investigated in studying the long-term effects of CR on aging. For obvious reasons, the CR animals weigh less and have less body fat. These effects were evident within 3 years of CR. CR monkeys have reduced trunk fat and demonstrate reduced levels of triglycerides and cholesterol and increased levels of high-density lipoproteins (HDLs). Furthermore, they have reduced fasting glucose and insulin levels as compared to controls, suggestive of alterations in glucoregulation. These data provided early evidence of the potential benefit of CR for the risk for diabetes and cardiovascular disease (Lane et al., 1999).

The Wisconsin Regional Primate Research Center initiated a sister study reporting the effects of long-term dietary restriction in male rhesus monkeys and later female monkeys. Gresl and colleagues (Gresl et al., 2001) studied the effects of moderate dietary restriction (70% baseline intake) after 8.5 years (beginning at 8-14 years of age). These investigators confirmed earlier reports chronic dietary restriction has protective effects against the development of insulin resistance as well as improved glucoregulatory parameters in aging rhesus monkeys.

Bone loss with aging is an important health concern and macaques have been shown to be an appropriate model in which to study skeletal effects of aging with regard to menopause (Black et al., 2001; Colman et al., 1999). Body weight and lean mass are related to bone mineral density and bone mineral content in multiple sites. Consequently, CR monkeys display reduced bone mass in specific sites; however, calcium homeostasis and bone turnover are not different from control animals. Therefore, although CR does not appear to retard or reverse age-associated bone, neither does it have negative effects on the system (Lane et al., 2001).

Calorie Restriction and Ovarian Aging

Extension of ovarian lifespan has been well characterized in the rodent, using CR protocols. Although the exact mechanisms of action explaining how this occurs is still largely unknown, it is believed to act by altering and/or improving the function of a variety of physiological systems. Reproductive

studies in CR rodents have also been performed. Rats maintained at 50% body weight (as compared to control littermates) still achieved sexual maturation, albeit delayed (Merry and Holehan, 1979). Onset of puberty in CR rats was observed once animals reached body weights similar to pubertal controls (Holehan and Merry, 1985c). The onset of reproductive decline, however, was significantly delayed as well (Holehan and Merry, 1985c; Merry and Holehan, 1979). Nelson et al. (Nelson et al., 1985) evaluated ovarian reserve in CR (alternate day fasting) mice. The investigators found that in mice, CR suppressed estrous cyclicity, and a return to ad libitum feeding restored cyclicity. Histological data demonstrated that CR rats had twice the number of primordial follicles as their age-matched controls. Furthermore, CR mice maintained cyclicity at an age when their age-matched controls were 80% acyclic. Therefore, calorie restriction may delay reproductive senescence either by delaying puberty or initiating a period of ovarian “rest.”

More recent data suggest that caloric restriction affects reproductive longevity at the level of the hypothalamus and/or pituitary (McShane and Wise, 1996). Investigators have found that female rats restricted to 60% of ad libitum feeding after the onset of puberty did not experience any interruption of normal cycling, but delayed cessation of estrous cycles was still observed. Therefore, they concluded that caloric restriction affected the reproductive system in rats by a mechanism other than simply delaying puberty or disrupting normal cycling. McShane and Wise (McShane and Wise, 1996) hypothesize that caloric restriction may actually preserve the reproductive neuroendocrine axis, allowing

for prolonged reproductive ability in these animals. In control animals, LH concentration and pulse amplitude decline with age; however, CR animals demonstrated enhanced LH secretion and this may be attributable to some enhanced pituitary or hypothalamic factor(s). It was suggested that neuropeptide Y could be involved in such effects, since it has been shown to increase during periods of food restriction.

Due to the apparent success in delaying senescence in rodents, work is currently being undertaken to study the effects of caloric restriction in the non-human primate. Experiments have been conducted to assess the issue of bone loss with respect to energy restriction. Rhesus monkeys experience menopause similar to humans and as such are a good model for assessing age-related bone loss (Colman et al., 1999). Data showed that moderate (30%) long-term energy restriction does not affect bone mineral density, bone mineral content, osteocalcin, 25-hydroxyvitamin D or parathyroid hormone concentrations (Lane et al., 2001). Therefore, the impact for use of caloric restriction without consequence to bone mass may be seriously considered. Investigators also assessed reproductive health in these monkeys and documented no interruption of regular cyclicity. Results suggest that energy restriction did not negatively affect the monkeys; however, they did not appear to exhibit any retardation of age-related changes either. Recent evidence in young, short-term (three months) CR rhesus monkeys also showed evidence of continued cycling; however, data on older monkeys have yet to be determined. Thus far, the data suggest that energy restriction is not overly traumatic to the reproductive system;

however, effects of CR on normal reproductive parameters are still not well studied. Lane *et al.* (Lane et al., 2001) did not detect any CR-related differences in the reproductive cycling of young and menopausal animals; however, there was little information regarding the peri-menopausal period, which is a critical transition period. Therefore, more studies need to be completed to fully understand the potential impact of CR on female reproduction.

SUMMARY

Clinical menopause is defined as the period of a woman's life one year after the cessation of menstrual cycles, however, the events leading to the climacteric begin years earlier and are termed the peri-menopausal transition. Data collected by the Center for Disease Control (CDC) have shown that there is a significant age-related decrease in the incidence of pregnancy and age-related increases in spontaneous miscarriage, ectopic pregnancies and chromosomal abnormalities in young women (<30 years;(Ventura et al., 2004). This is a forbidding reality, especially with an increasing number of women choosing to start their careers before starting families (te Velde et al., 1998). The obvious impact of potential infertility in young women notwithstanding, the long-term consequences of menopause cannot be dismissed. The average age of menopause in women is approximately 51 years, resulting in a post-reproductive period that extends for nearly 1/3 of a woman's life (Treloar, 1981). Multiple non-reproductive systems are affected by the sudden withdrawal of hormonal support that is associated with menopause, including bone and cardiovascular health

(Gosden, 1985; Prior, 1998). Menopause is a biological inevitability for all women, yet little is known about its progression. There is a great need to develop useful models and indices to mark the progression of the peri-menopause. Furthermore, research into methods by which menopause may be delayed, such as calorie restriction (or CR mimetics) would be extremely beneficial and timely.

The collaborative effort between the National Institute on Aging, the Oregon Health and Sciences University (OHSU) and the University of Maryland has made possible a comprehensive analysis of the effects of moderate CR in both short- and long-term regimens, on reproductive function in young and peri-menopausal rhesus monkeys. Therefore, the objectives of this project were to: 1) analyze general hormonal changes that occur with aging and menopause and 2) evaluate the effects (whether beneficial or detrimental) of moderate (30%) CR on ovarian function and decline in rhesus monkeys.

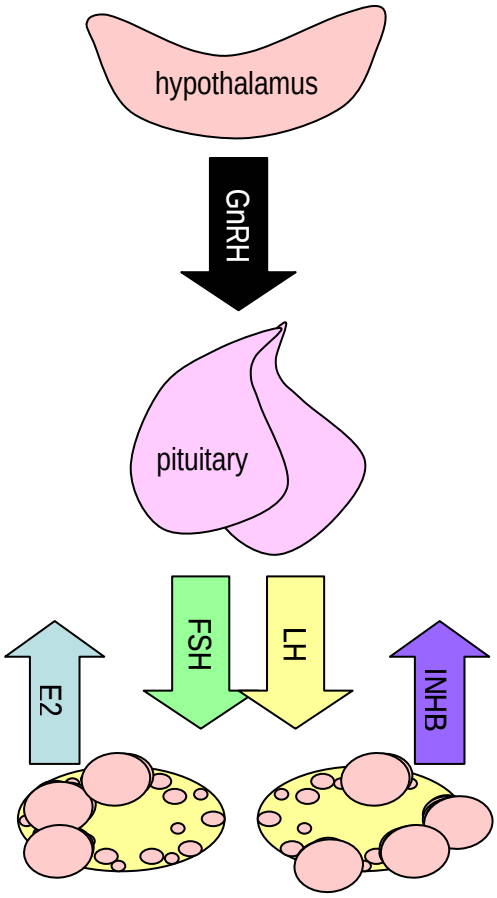


Figure 1.1 Hypothalamic Pituitary Gonadal Axis. Schematic representation of the hypothalamic pituitary gonadal axis and the major hormones involved. Hypothalamic production of gonadotropin releasing hormone (GnRH) stimulates pituitary production of gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Ovarian response to gonadotropins stimulated the release of estradiol (E2) and inhibin B (INHB).

Table 1.1 Summary of evolutionary hypotheses and theories of menopause.

Evolutionary Hypothesis or Theory	Description
Good Mother Hypothesis	Risk of pregnancy resulting in a trade-off between an increased number of children and ability to successfully raise them (Alexander, 1974; Nesse and Williams, 1996; Sherman, 1998)
Grandmother Hypothesis	Re-allocation of reproductive effort from production of own offspring to caring for offspring already born and/or children's offspring (Gibbons, 1997; Hawkes et al., 1998; Williams, 1957)
Disposable Soma Theory	Favoring of reproductive systems over maintenance and repair systems; menopause phenotype appearing with increased lifespan (Kirkwood, 2002)

Table 1.2 Summary of Research Models of Ovarian Aging and Menopause

Animal	Model	Phenotype
Rodent	Mouse / Rat	Normal ovarian senescence: ~6-18 months (Felicio et al., 1984; Johnson, 2000b; Lu et al., 1979; vom Saal et al., 1994)
	Calorie Restricted Rat	Delayed ovarian senescence (Holehan and Merry, 1985b; Merry and Holehan, 1979; Nelson et al., 1985)
	Bax $^{-/-}$ Mouse	Delayed ovarian senescence (Hsu et al., 1997; Kim, 2005; Kugu et al., 1998; Liu et al., 1996; Oltvai et al., 1993; Sedlak et al., 1995; Tilly et al., 1995; Yang et al., 1995)
	VCD-Treated Mouse/Rat	Accelerated ovarian senescence (Borman et al., 1999; Doerr et al., 1995; Hooser et al., 1994; Hoyer et al., 2001; Hu et al., 2001; Mayer et al., 2002; Springer et al., 1996)
	Foxo3a$^{-/-}$ Mouse	Accelerated ovarian senescence (Brenkman and Burgering, 2003; Burgering and Medema, 2003; Castrillon et al., 2003; Hosaka et al., 2004; Kenyon, 2001; Kenyon et al., 1993; Tran et al., 2003)
	FSH-R$^{+/-}$ Mouse	Accelerated ovarian senescence (Billig et al., 1994; Camp et al., 1991; Danilovich et al., 2002; Danilovich and Sairam, 2002; Dierich et al., 1998; Gosden et al., 1983; Hillier, 2001; Zeleznik et al., 1974)
Primate	Human	Normal ovarian senescence: ~50 years (Abma et al., 1997; Akande et al., 2004; Bukman and Heineman, 2001; Chin et al., 2002; Navot et al., 1994; Treloar, 1981; Ventura et al., 2004; Vollman, 1977)
	Rhesus Monkey	Normal ovarian senescence: ~24 years (Gilardi et al., 1997; Heape, 1900; Shideler et al., 2001)

CHAPTER 2

MENSTRUAL CYCLING WITH PERI-MENOPAUSE IN RHESUS MONKEYS AND THE EFFECTS OF CALORIE RESTRICTION

INTRODUCTION

Calorie restriction (CR) is a paradigm of life extension that was first reported in the mid 1930's (McCay et al., 1935). It remains the only proven non-genetic and nutritional experimental paradigm for life extension, retarding aging processes in many species, including rats, mice, fish, hamsters, fruit flies and nematodes (Roth et al., 1999; Sinclair, 2005; Smith et al., 2004). Its long history notwithstanding, very little is known with regard to the mechanism of action of CR. Its effects on age-related illnesses are currently being investigated. Furthermore, moderate CR in rats has been shown to delay the onset of ovarian decline (Holehan and Merry, 1985b; Merry and Holehan, 1979).

The median age of menopause in women is 51 years (Treloar, 1981). The associated health risks that accompany the climacteric are well known, including cardiovascular, cognitive and skeletal diseases (Johnson et al., 2004; Nappi et al., 1999; Prior, 1998; Sherwin, 2003). Despite this, there remains only a modest understanding of how and why menopause occurs. Use of potentially inappropriate animal models (species that do not have a "true" menopause) may also confuse the science behind the precipitous decline reproductive function in women (Wu et al., 2005).

The rhesus monkey is an ideal animal model in which to study ovarian function and decline in women. Rhesus monkeys exhibit menstrual cycles comparable to women, both hormonally and with regard to the endometrial sloughing of the uterine lining (Heape, 1900). Limited data have demonstrated that rhesus monkeys undergo menopause similar to women at approximately 25 years (Gilardi et al., 1997). Morphometric follicular counting techniques have also been used to characterize changes in ovarian reserve (follicular number) in both pigtail and rhesus macaques (Miller et al., 1999; Nichols et al., 2005). Unlike other laboratory animal models, such as the rat and mouse, rhesus monkeys may be monitored for cessation of menstruation (true menopause) as is practiced in human clinics.

In rats, moderate CR delays the onset of ovarian decline, typical in aging females (Holehan and Merry, 1985b; Merry and Holehan, 1979). The effects of CR in primates are largely unknown (Mattison et al., 2003); however, we previously reported that CR did not appear to negatively impact menstrual cycles or hormone profiles in our rhesus monkeys (Wu et al., 2004). The effects of CR on delaying menopause, however, have yet to be determined. A controlled delay of menopause could beneficially impact multiple systems beyond reproduction, including a reduction in the incidence of cardiovascular disease, bone fragility, cognitive deficits and certain cancers. Therefore the objective of this experiment was two-fold: 1) to evaluate hormone changes that occur with normative aging and onset of peri-menopause; and 2) to determine what effects (either beneficial or detrimental) CR has on menstrual cycling in rhesus monkeys.

MATERIALS AND METHODS

Animals and Diet

Adult female rhesus monkeys *(Macaca mulatta)*, exhibiting normal body weights (4.5-7.0 kg) and regular menstrual cycles of about 28 days were housed under controlled conditions of temperature (22°C) and a standard daily light-dark cycle (12 h light: 12 h dark). The veterinary staff provided animal care and housing. Additionally, all procedures were approved by the Institutional Animal Care & Use Committee (IACUC) at the Oregon National Primate Research Center and the Animal Care and Use Committee (ACUC) of the NIA, in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Body weights of each animal were assessed monthly. Two colonies of monkeys were used in this study: 1) National Institute on Aging (NIA; Poolesville, MD), 2) Oregon National Primate Research Center (ONPRC), Oregon Health and Sciences University (OHSU; Beaverton, OR).

Prior to implementation of the CR feeding regimen, monkeys were monitored to determine daily food intake based on *ad libitum* feed availability. The average daily value was then divided into two meals. Feed allotment was adjusted for young, growing monkeys maintained in the long-term study. CR monkey diet was reduced by 10% per month until a 30% CR was achieved.

Short-Term (ONPRC) Colony: Monkeys housed at the ONPRC colony were maintained on the CR diet for 1-2 years. Breakdown of animal groups are as follows in Table 2.1.

Table 2.1 Short-term (ONPRC) Animal Distribution. Animal ages and numbers for the colony housed at the Oregon National Primate Research Center (ONPRC). The calorie restriction (CR) protocol was maintained for 1-2 years.

ONPRC	Age*	N
YCON	10 ± 0	4
YCR	10 ± 0	5
OCON	23 ± 0.9	8
OCR	22 ± 0.8	7

*Age in years at time of sampling

Long-Term (NIA) Colony: Monkeys housed at the NIA colony have been maintained on the CR diet for 11-12 years. Breakdown of animal groups are as follows in Table 2.2.

Table 2.2 Long-term (NIA) Animal Distribution. Animal ages and numbers for the colony housed at the National Institute on Aging (NIA). The calorie restriction (CR) protocol was maintained for 11-12 years.

NIA	Age*	N
YCON	14 ± 0.3	6
YCR	14 ± 0.2	7
OCON	20 ± 1.1	6
OCR	20 ± 1.5	5

*Age in years at time of sampling

Blood Collection and Hormone Analyses:

Monkeys were monitored for onset of menses (designated Menses d1). Serum was collected from un-anesthetized monkeys from the saphenous vein for 3 cycles (or approximately 90 days) following a spontaneous menses: daily during the follicular phase and every third day during the luteal phase. Circulating hormones were assayed for estradiol (E2) and progesterone (P4) by specific electrochemoluminescent assay using a Roche Elecsys 2010 assay instrument at the Endocrine Services Laboratory Core, ONPRC (Beaverton, OR). Luteinizing hormone (LH) was determined using a Leydig cell bioassay and follicle-stimulating hormone (FSH) was determined using an in-house

radioimmunoassay (RIA). Hormone concentrations were validated against previous RIAs in this laboratory (Hess et al., 1981; Zelinski-Wooten et al., 1995).

Inhibin B was determined using the commercially available Active Inhibin B kit (Diagnostic Systems Laboratory; Webster, TX). This ELISA was validated for use with rhesus monkey serum in our laboratory. Parallel displacement curves were obtained by comparing serially diluted (1:1 to 1:16) pooled rhesus monkey serum against known INHB concentrations. Recovery of known concentrations of unlabeled INHB added to pooled rhesus monkey serum was 100% with a coefficient of variation of 10%. Inter-assay coefficients of variation for two internal controls were 15% and 9%. The intra-assay coefficient of variation was always less than 10%.

Hormone Profiles Relative to Mid-Cycle LH peak

All hormones (LH, FSH, E2, P4 and INHB) were retrospectively synchronized relative to the estimated mid-cycle LH peak (designated LH d0). This separates the follicular and luteal phases to permit analyses between comparable physiological states. Monkeys were assigned to one of two groups: regular cyclers (≥2 cycles demonstrating a mid-cycle E2 peak followed by luteal phase levels of P4) and irregular cyclers). Hormone levels were averaged for each female exhibiting regular cycles across the three consecutive cycles and subsequently averaged within age and diet groups (YCON, YCR, OCON, OCR). Irregular cyclers could not be included in these analyses due to lack of information (i.e. LH peak).

Hormone Profiles Relative to the Onset of Menses

A second analysis was performed using early follicular phase hormones following onset of menses (designated Menses d1). E2, FSH and INHB were aligned by Menses d1. Based on the classification of regular and irregular cyclers, defined above, monkeys were divided into 6 groups: YCON, YCR, OCON Regular Cyclers (OR-CON), OCR Regular Cyclers (OR-CR), OCON Irregular Cyclers (OI-CON) and OCR Irregular Cyclers (OI-CR). In specific cases where menses did not occur, nadirs (< 50 pg/mL) in E2 patterns were used and assigned as d1.

Short-Term (ONPRC): Menses Records in the Third Year

These data were collected as part of a larger experiment which continued into a third year on CR. Subsequent to our 90-day sampling period (performed after 1-2 years on CR), record-keeping on menses events persisted until the completion of the experiment at three years. Using Gilardi and colleagues' (Gilardi et al., 1997) definition, we classified a monkey as an irregular cycler if it exhibited 6 consecutive months or more of irregular cycles, excluding the non breeding season (June through August).

Statistical Analysis

Data are expressed as mean ± SEM for each parameter measured in each group. Standard descriptive statistics were performed. Log transformation of hormones was necessary and homogeneity of variances and normality of

residuals were tested. Hormones were analyzed by 2-way ANOVA repeated measures, followed by Tukey's for post-hoc comparisons (SAS; Cary, NC) and significant differences were established at $p < 0.05$.

RESULTS

Short-Term (ONPRC): Individual Hormone Profiles

Figures 2.1 depicts representative estradiol (E2) and progesterone (P4) graphs for three consecutive menstrual cycles in regular cyclers for YCON (A), YCR (B), OCON (C) and OCR (D) monkeys housed for short-term at the ONPRC facility. Regular cyclers included monkeys exhibiting ≥ 2 cycles with a mid-cycle E2 peak followed by luteal phase levels of P4. All of the young monkeys were categorized as regular cyclers, while five of eight OCON and five of seven OCR were classified as regular cyclers (Table 2.3).

Table 2.3 Distribution of Regular and Irregular Cyclers in Old Short-Term (ONPRC) Group. Regular cyclers included monkeys exhibiting ≥ 2 cycles with a mid-cycle E2 peak followed by luteal phase levels (> 1 ng/mL) of P4. All young monkeys cycled regularly.

ONPRC	Regular Cyclers	Irregular Cyclers	Total
OCON	5	3	8
OCR	5	2	7

Figure 2.2 depicts individual E2 and P4 graphs for approximately 90 days in OCON Irregular Cyclers housed short-term at the ONPRC facility. Three of eight OCON exhibited irregular cycles. In figure 2.2A, no menses data were collected over the course of the 90-day observation period. A single mid-cycle E2 peak occurred, followed by normal luteal phase levels of P4. In Figure 2.2B, two menses incidences were recorded, although the first incident may not be

considered a “true” menses due to the lack luteal phase levels of P4 preceding the event. This is an example of break through bleeding (BTB) which occurs following an extended period of E2-induced endometrial proliferation. In Figure 2.2C, the monkey displayed very low levels of E2 and P4, comparable to expected hormone profiles from an ovariectomized animal, with no recorded menses activity and little to no change in both E2 and P4.

Figure 2.3 depicts individual E2 and P4 graphs for approximately 90 days in OCR Irregular Cyclers maintained on short-term CR (1-2 years) at the ONPRC facility. Two of seven OCR exhibited irregular cycles. Figure 2.3A presents another example of very low hormone activity, with no recorded menses noted and little to no change in both E2 and P4. Hormone levels in Figure 2.3B indicate that the monkey was mid-luteal when blood sampling was initiated. Three menses events were recorded and a single normal cycle occurred (with a mid-cycle E2 peak and luteal phase levels of P4). The third menses event may not be considered a “true” menses due to the lack of luteal phase levels of P4 preceding the menses and is likely due to BTB.

Short-Term (ONPRC): Mean Hormone Profiles

Figure 2.4 depicts mean hormone levels for each female exhibiting regular cycles across three consecutive menstrual cycles and normalized to the estimated mid-cycle LH peak (d=0). As described above, only regular cyclers were included in these analyses. All YCON (n=5) and YCR (n=5) were included while 5 of 8 OCON and 5 of 7 OCR were included (Table 2.3). Due to financial

limitations, selected days were chosen for hormone analyses for LH (three days before and after the expected LH peak), FSH (follicular phase) and INHB (follicular phase). A summary of the statistical differences is outlined in Table 2.4.

Table 2.4 Short-Term (ONPRC) Summary Statistics Relative to LH Peak. Summary of statistical analyses for mean hormone profiles normalized relative to estimated LH peak (d=0). Differences ($p < 0.05$) are indicated in bold. Individual comparisons were not applicable (NA) when overall differences were not observed. ">" or "<" indicates the relative difference between the groups under comparison.

Comparison	LH	FSH	E2	P4	INHB
Y vs. O	<	<	=	=	>
CON vs. CR	NA	>	=	=	=
YCON vs. YCR	NA	=	NA	NA	NA
YCON vs. OCON	NA	<	NA	NA	NA
YCON vs. OCR	NA	=	NA	NA	NA
YCR vs. OCON	NA	<	NA	NA	NA
YCR vs. OCR	NA	=	NA	NA	NA
OCON vs. OCR	NA	=	NA	NA	NA

Figure 2.4A depicts mean LH levels for regularly cycling YCON, YCR, OCON and OCR monkeys. Young monkeys had significantly lower LH levels as compared to Old monkeys, however no diet effects were observed. Figure 2.4B depicts mean FSH levels. Young monkeys had significantly lower FSH levels as compared to Old monkeys. CON monkeys also displayed higher FSH levels compared to CR monkeys. Figure 2.4C-D depicts mean E2 and P4 levels, respectively. Both E2 and P4 levels were similar between ages and diet. Figure 2.4E depicts mean INHB levels. Young monkeys had significantly higher INHB levels as compared to Old monkeys, and no diet effects were observed.

Figure 2.5 and Figure 2.6 depict mean hormone levels for each female, averaged across three consecutive menstrual cycles or approximately 90 days and normalized to the onset of menses or nadirs (<50 pg/mL) in E2 patterns (designated Menses d1). Regular and irregular cyclers were classified as defined above. All Young monkeys cycled regularly; however, Old monkeys were divided into either Old, Regular (OR-CON, OR-CR) or Old, Irregular (OI-CON, OI-CR). Clinical data in humans demonstrate the early follicular phase to be temporally more sensitive in identifying differences between ages. Therefore, we focused on Menses d1-d5. A summary of the statistical differences is outlined in Table 2.5.

Table 2.5 Short-Term (ONPRC) Summary Statistics Relative to Onset of Menses. Summary of statistical analyses for mean hormone profiles with comparisons on Menses d1-d5. Differences ($p < 0.05$) are indicated in bold. ">" or "<" indicates the relative difference between the groups under comparison.

Comparison	FSH	INHB
Y vs. O	<	>
CON vs. CR	=	=
Y vs. OR	<	>
Y vs. OI	=	>
OR vs. OI	>	>

Figure 2.5 depicts FSH levels for YCON, YCR, OR-CON, OR-CR, OI-CON and OI-CR on menses d1-d5. Young monkeys had significantly lower FSH levels than Old monkeys; however there were no diet effects. OR monkeys had the highest levels of FSH, while FSH in OI and Y monkeys were similar.

Figure 2.6 depicts INHB levels for YCON, YCR, OR-CON, OR-CR, OI-CON and OI-CR on menses d1-d5. Young monkeys had significantly higher

INHB levels than Old monkeys, with no diet effects. OI monkeys had the lowest levels of INHB, with OR monkeys intermediate and Y monkeys highest.

Long-Term (NIA): Individual Hormone Profiles

Figure 2.7A-D depicts representative estradiol (E2) and progesterone (P4) graphs for three consecutive menstrual cycles in regular cyclers for YCON (A), YCR (B), OCON (C) and OCR (D) monkeys housed for long-term at the NIA facility. Regular cyclers included monkeys exhibiting ≥ 2 cycles with a mid-cycle E2 peak followed by luteal phase levels of P4. All of the young monkeys were categorized as regular cyclers, while three of six OCON and one of five OCR were classified as regular cyclers (Table 2.6).

Table 2.6 Distribution of Regular and Irregular Cyclers in Old Long-Term (NIA) Group. Regular cyclers included monkeys exhibiting ≥ 2 cycles with a mid-cycle E2 peak followed by luteal phase levels (> 1 ng/mL) of P4. All young monkeys cycled regularly.

NIA	Regular Cyclers	Irregular Cyclers	Total
OCON	3	3	6
OCR	1	4	5

Figure 2.8 depicts individual E2 and P4 graphs for approximately 90 days in OCON Irregular Cyclers housed for long-term at the NIA facility. Three of six OCON exhibited irregular cycles. In figure 2.8A, blood collections were started just prior to the mid-cycle E2 peak, followed by a normal luteal phase levels of P4 and subsequent menses. No further menses data were collected, and E2 was normal for basal levels, although no further peaks or luteal phases were observed. Figure 2.8B presents another example of extremely low levels of hormones, with an absence of hormone cycling. Although no E2 peak was

detected, low, normal P4 luteal phase levels were observed. Figure 2.8C shows a singe normal cycle with a record of menses followed by a mid-cycle E2 peak and luteal phase levels of P4. This was followed by a period of very low levels of hormone activity and no further menses observations.

Figure 2.9 depicts individual E2 and P4 graphs for approximately 90 days in OCR Irregular Cyclers housed on long-term CR (11-12 years) at the NIA facility. Four of five OCR exhibited irregular cycles. Figure 2.9A shows three recorded menses; however, the second incident is likely break through bleeding (BTB), due to the absence of a preceding luteal phase. E2 appeared to peak initially but failed to induce ovulation and luteal production of P4. This was followed by a normal cycle. Figure 2.9B shows cycling E2 levels with no peaks, ovulations or documented menses. Figure 2.9C also shows cycling E2 levels, with no ovulations; however, multiple menses events were recorded. These are likely a product of BTB. Figure 2.9D shows a mid-cycle E2 peak that was followed by very low levels of P4 and subsequent menses. This was followed by cycling levels of E2 and another peak that was followed by very low levels of P4.

Long-Term (NIA): Mean Hormone Profiles

Figure 2.10 depicts mean hormone levels for each female exhibiting regular cycles, averaged across three consecutive menstrual cycles and normalized to the estimated mid-cycle LH peak (d=0). As described above, only regular cyclers were included in these analyses. All YCON (n=6) and YCR (n=7) were included while 3 of 6 OCON and 1 of 5 OCR were included. Selected days

were chosen for hormone analyses for LH (three days before and after the expected LH peak), FSH (follicular phase) and INHB (follicular phase). A summary of the statistical differences is outlined in Table 2.7.

Table 2.7 Long-Term (NIA) Summary Statistics Relative to LH Peak. Summary of statistical analyses for mean hormone profiles normalized relative to estimated LH peak (d=0). Individual comparisons were not applicable (NA) when overall differences were not observed. ">" or "<" indicates the relative difference between the groups under comparison.

Comparison	LH	FSH	E2	P4	INHB
Y vs. O	=	=	=	=	=
CON vs. CR	=	=	=	=	=
YCON vs. YCR	NA	NA	NA	NA	NA
YCON vs. OCON	NA	NA	NA	NA	NA
YCON vs. OCR	NA	NA	NA	NA	NA
YCR vs. OCON	NA	NA	NA	NA	NA
YCR vs. OCR	NA	NA	NA	NA	NA
OCON vs. OCR	NA	NA	NA	NA	NA

Figures 2.10A-E depict mean LH, FSH, E2, P4 and INHB levels for regularly cycling YCON, YCR, OCON and OCR monkeys. Hormones were similar between ages and diets for all hormones.

Figure 2.11 and Figure 2.12 depict mean hormone levels for each female, averaged across three consecutive menstrual cycles or approximately 90 days and normalized to the onset of menses or nadirs (<50 pg/mL) in E2 patterns (designated Menses d1). Regular and irregular cyclers were classified as defined above. All Young monkeys cycled regularly; however, Old monkeys were divided into either Old, Regular (OR-CON, OR-CR) or Old, Irregular (OI-CON, OI-CR) groups. Focusing again on Menses d1-d5, a summary of the statistical differences is outlined in Table 2.8.

Table 2.8 Long-Term (NIA) Summary Statistics Relative to Onset of Menses. Summary of statistical analyses for mean hormone profiles with comparisons on Menses d1-d5. Differences ($p < 0.05$) are indicated in bold. ">" or "<" indicates the relative difference between the groups under comparison.

Comparison	FSH	INHB
Y vs. O	<	>
CON vs. CR	=	=
Y vs. OR	=	=
Y vs. OI	<	>
OR vs. OI	<	>

Figure 2.11 depicts FSH levels for YCON, YCR, OR-CON, OR-CR, OI-CON and OI-CR on menses d1-d5. Young monkeys had significantly lower FSH levels than Old monkeys, however there were no diet effects. Young and OR FSH levels were similar, however, Young monkeys had lower FSH levels as compared to OI monkeys. Additionally, OR monkeys had lower FSH levels than OI monkeys.

Figure 2.12 depicts INHB levels for YCON, YCR, OR-CON, OR-CR, OI-CON and OI-CR on menses d1-d5. Young monkeys had significantly higher INHB levels than Old monkeys, with no diet effects. As with FSH levels, Young and OR monkeys had similar INHB levels. Young monkeys had higher INHB levels than OI; OR monkeys had higher INHB levels than OI as well.

Short-Term (ONPRC): Menses Records in the Third Year

Short-term (ONPRC) monkeys were monitored for menses into the third year on CR. Table 2.9 shows the distribution of regular and irregular cyclers of the old short-term (ONPRC) monkeys. Previously determined (based on 90 day hormone data; Table 2.3) numbers of regular and irregular cyclers are indicated

in parentheses. The relative number of irregular cyclers in the OCR group increased (from 2 of 7 to 5 or 7) while the number of irregular cyclers in the OCON group remained the same (3 of 8).

Table 2.9 Distribution of Regular and Irregular Cyclers in the Old Short-Term (ONPRC) Group in Year 3. Using Gilardi and colleagues' (Gilardi et al., 1997) definition for regular and irregular cyclers, menses records were used to evaluate menstrual cyclicity in the third year on CR. Parenthesized numbers indicate regular and irregular cycler numbers as determined after 1-2 years on CR (Table 2.3).

ONPRC	Regular Cyclers	Irregular Cyclers	Total
OCON	4 (5)	3 (3)	7 (8)
OCR	2 (5)	5 (2)	7 (7)

DISCUSSION:

Menopause is not strictly a reproductive issue. Its effects cross over into other areas of health and well-being, including cognitive, skeletal and cardiovascular systems (Johnson et al., 2004; Nappi et al., 1999; Prior, 1998; Sherwin, 2003). Therefore, the need to find an appropriate model in which to study this inevitable life event is extremely relevant and emergent. Furthermore, the development of potential interventions (i.e. calorie restriction) by which to delay menopause would be extraordinarily beneficial.

The Rhesus Monkey as a Model for Menopause

The hypothalamic-pituitary axis and the ovary act in concert to regulate all facets of female reproduction. At the time of reproductive decline, the hormones released by these feedback circuits are disrupted. Although FSH is elevated and INHB is reduced in women (Klein et al., 1996a; Soules et al., 1998), little

research has been performed investigating the endocrine patterns of peri-menopause in rhesus monkeys.

Researchers have recommended the use rhesus monkeys as an appropriate animal model for studying menopause (Bellino and Wise, 2003; Heape, 1900). Previous reports have also shown that like women, menopause in rhesus monkeys is associated with amenorrhea, low urinary estrone conjugates (which are metabolites of circulating estrogens), and irregular patterns of urinary pregnanediol-3 glucuronide (progesterone metabolites; (Gilardi et al., 1997). Using trained monkeys, we were able to collect serum over the course of approximately 90 consecutive days and confirmed similar changes to those observed in women: the absence of mid-cycle E2 peaks and the accompanying luteal phase, in older females. Older, peri-menopausal monkeys also displayed irregularly timed menses intervals.

Shideler, and colleagues (Shideler et al., 2001) reported that urinary FSH levels were elevated in menopausal rhesus monkeys only after menstrual irregularities were noted. These data were surprising given that in women, FSH is one of the first detectable signs of reproductive decline. Our data collected from the ONPRC colony provides the first evidence that rhesus monkeys do in fact have elevated FSH levels prior to exhibiting irregular menstrual cycles, with FSH in Y monkeys lower than OR. Surprisingly, OI monkeys had FSH levels similar to Y and less than OR monkeys. There are a couple of possible explanations for these observations. It may be that after a period of elevated FSH levels without appropriate ovarian response, the pituitary halted FSH

production in the OI females. Alternatively, it may have been due to stress-related shut down in the hypothalamic pituitary gonadal axis.

In the NIA monkeys, FSH levels were also lower among the Y as compared to O monkeys; however, there was no difference between FSH levels between Y and OR monkeys. This is likely due to the fact that the O monkeys in the NIA colony are on average 2-3 years younger than the ONPRC colony. During the peri-menopause a difference of a few years may be critical and sensitive time for hormone changes. As expected, however, OI monkeys displayed elevated FSH levels and were significantly different from both Y and OR monkeys.

INHB levels reflected the hormone changes that paralleled FSH. Interestingly, we observed a slight difference in the pattern of hormone distribution between the Y, OR and OI groups with INHB as compared to our FSH results. The INHB data were in fact more consistent with predicted changes in that Y monkeys exhibited the highest levels of INHB; OI monkeys had the lowest levels and OR monkeys were intermediate. The difference between the two hormones may be explained by the temporal relationship of the hormone changes. That is, our data are consistent with data from peri-menopausal women that suggest that changes in INHB occur not only prior to menstrual cycle irregularity, but also prior to changes in FSH.

Calorie Restriction and Menstrual Cycling

Nutrition and reproduction are exquisitely interconnected. Both anorexia and obesity can have detrimental effects on reproduction, inhibiting both menses and fertility (Bray, 1997; Messinis and Milingos, 1999; Misra et al., 2004). Moderate CR, is a well known nutritional intervention that extends overall life span in many species. Effects of CR on reproduction and reproductive life span have in rodents have yielded mixed results. This study is also the first to evaluate semi-longitudinal menstrual cycling in monkeys maintained on a CR diet. A single study showed similarities in E2 and FSH between CON and CR rhesus monkeys (Black et al., 2001), however in this study, only samples collected on Menses day 5 were assayed in these comparisons.

Our data were carefully collected and monkeys were trained to the blood sampling protocol to remove the necessity for anesthesia and to reduce stress. After analyzing the hormone levels collected during the 90 day sampling periods, we conclude that 30% CR is not detrimental to normal menstrual cycling either in short- or long-term protocols. We found no differences between regularly cycling monkeys in LH, FSH, E2, P4, or INHB (relative to the LH peak) within age groups in either the long- or short-term CR animals. Even in the potentially more sensitive FSH and INHB analyses (Menses d1 through d5), CON and CR results were similar and therefore did not detect any deleterious effects of CR.

Both the short-term (ONPRC) and long-term (NIA) colonies were maintained on moderate levels of calorie restriction, with supplementation for

vitamins and minerals. These monkeys are restricted, but not malnourished. Therefore, we conclude from theses data, that this permits normal functioning of the reproductive axis. Furthermore, given the comparison between hormone profiles within the OR and OI groups, all the females are transitioning into the peri-menopause similarly. Whether moderate CR in rhesus monkeys will be able to delay menopause remain to be seen, however we can be confident that this level of nutritional intervention does not appear to be harmful to reproductive function in young monkeys.

Finally, in evaluating the menses records collected from the short-term (ONPRC) old females, we found that by year 3, changes in the relative numbers of irregular cyclers increased. There may be due to a trend for more monkeys to enter into peri-menopause in the short-term (ONPRC) CR group relative to CON. This may be a indication of advancement into peri-menopause by the CR group. The long-term (NIA) group of monkeys has been maintained on CR for 11-12 years, far longer than the short-term (ONPRC) group and yet we did not detect any difference between the Old monkeys. Initiation of CR later in life may not show detriments within the first couple of years, but later demonstrated signs of impaired ovarian function. The long-term (NIA) old monkeys are several years younger than the short-term (ONPRC) group; thus, it may be necessary to wait to determine if there will be similar changes and/or possible detriments to reproductive function with longer term application of moderate CR.

SUMMARY

Menopause is universally relevant, affecting all women at some point in their lives. Along with reduced fertility, menopause brings with it, many other physiological health risks. Despite the significance of this life event, little is known about the initiating triggers for menopause.

Our data provide extremely useful information with regard to the use of rhesus monkeys in menopause research. We have confirmed previous reports that rhesus monkeys exhibit similar menstrual cycles (approximately 28 days in length), and hormone profiles in young, regularly cycling animals. Additionally, we report for the first time that rhesus monkeys display reduced levels of INHB and elevated levels of FSH prior to menstrual cycle irregularities, similar to women. These data further confirm the suitability of the rhesus monkey model in ovarian aging research.

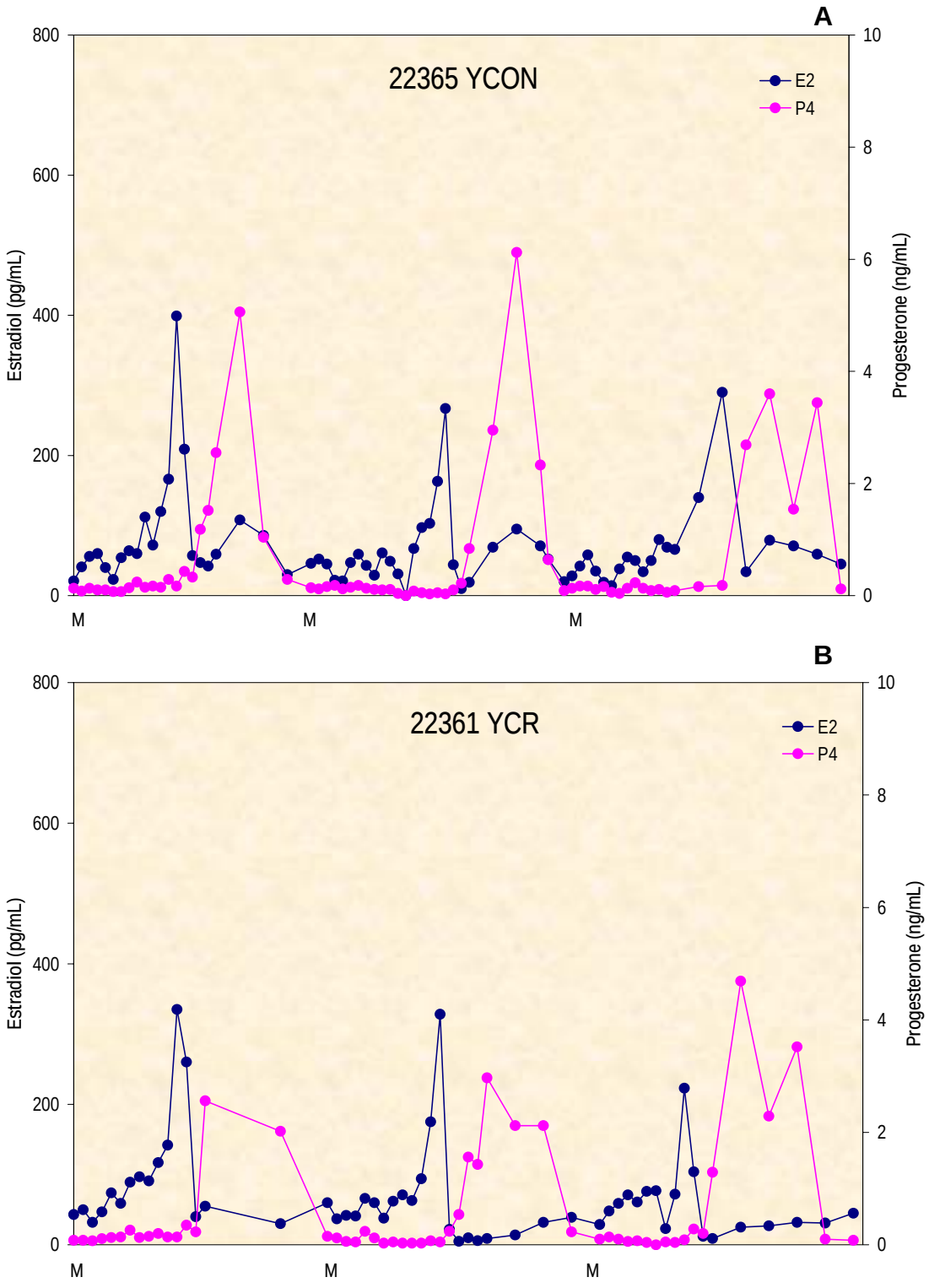
Finally, we demonstrated that moderate calorie restriction is not detrimental to normal ovarian cyclicity in young females. Initiation of moderate CR in old females, under short-term conditions, may have no impact within the first 1-2 years; however, some detrimental effects were observed after 3 years on the treatment in these old monkeys. There is a trend to advance cycle irregularity among old short-term (ONPRC) females. Long-term (NIA) old CR monkeys appear to be going through the normal progression of ovarian aging. This group of monkeys is several years younger and we will have to wait to see if they continue to function similarly to the old CON, or if they also begin to show

advanced signs of menstrual cycle irregularity. Furthermore, we are currently reviewing menses records to evaluate these females over the course of the full year, and not simply the 90-day period in which we collected samples.

It is therefore possible to pursue other possible benefits of CR without compromising reproductive function in young females. Young female rhesus monkeys continue to cycle normally on the nutritional protocol (both long- and short-term). To date, the long-term (NIA) older monkeys are displaying normal age-related hormonal changes as well. Given our results in the short-term (ONPRC) old monkeys, considerations must be made with regard to animal age at CR initiation and duration of the treatment.

ACKNOWLEDGMENTS

This research was support by NIH AG21382-01 and OHSU RR00163. We would like to thank the members of the Oregon National Primate Research Center (ONPRC): Diana Takahashi, Maralee Lawson, Dr. David Hess, Dr. Frank Koegler, Dr. Francis Pau, Dr. John Fanton, Dr. Ted Hobbs, Megan Martin, Lindsay Pranger and Olivia Thoene. We would also like to thank the members of the National Institute on Aging Animal Facility: Dr. Julie Mattison, April Handy, Ed Tilmont, Thomas Thompson, Jennifer Young, Melissa Lorence, Karl Reinsfelder, Dr. Douglas Powell and Dr. Rick Herbert.


A
22365 YCON
E2
P4
800
600
400
200
0
10
8
6
4
2
0
Estradiol (pg/mL)
Progesterone (ng/mL)
M
M
M
B
22361 YCR
E2
P4
800
600
400
200
0
10
8
6
4
2
0
Estradiol (pg/mL)
Progesterone (ng/mL)
M
M
M

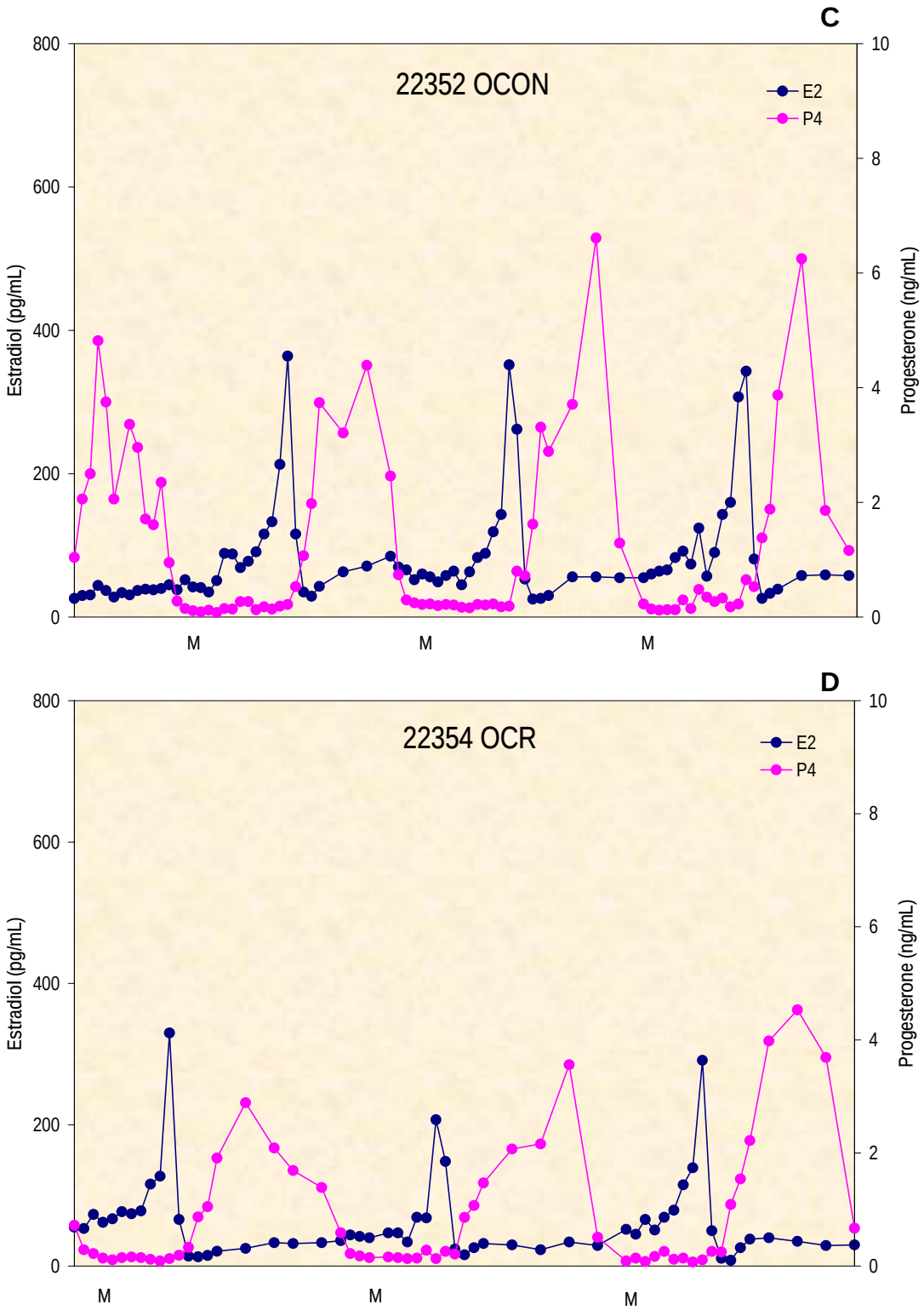
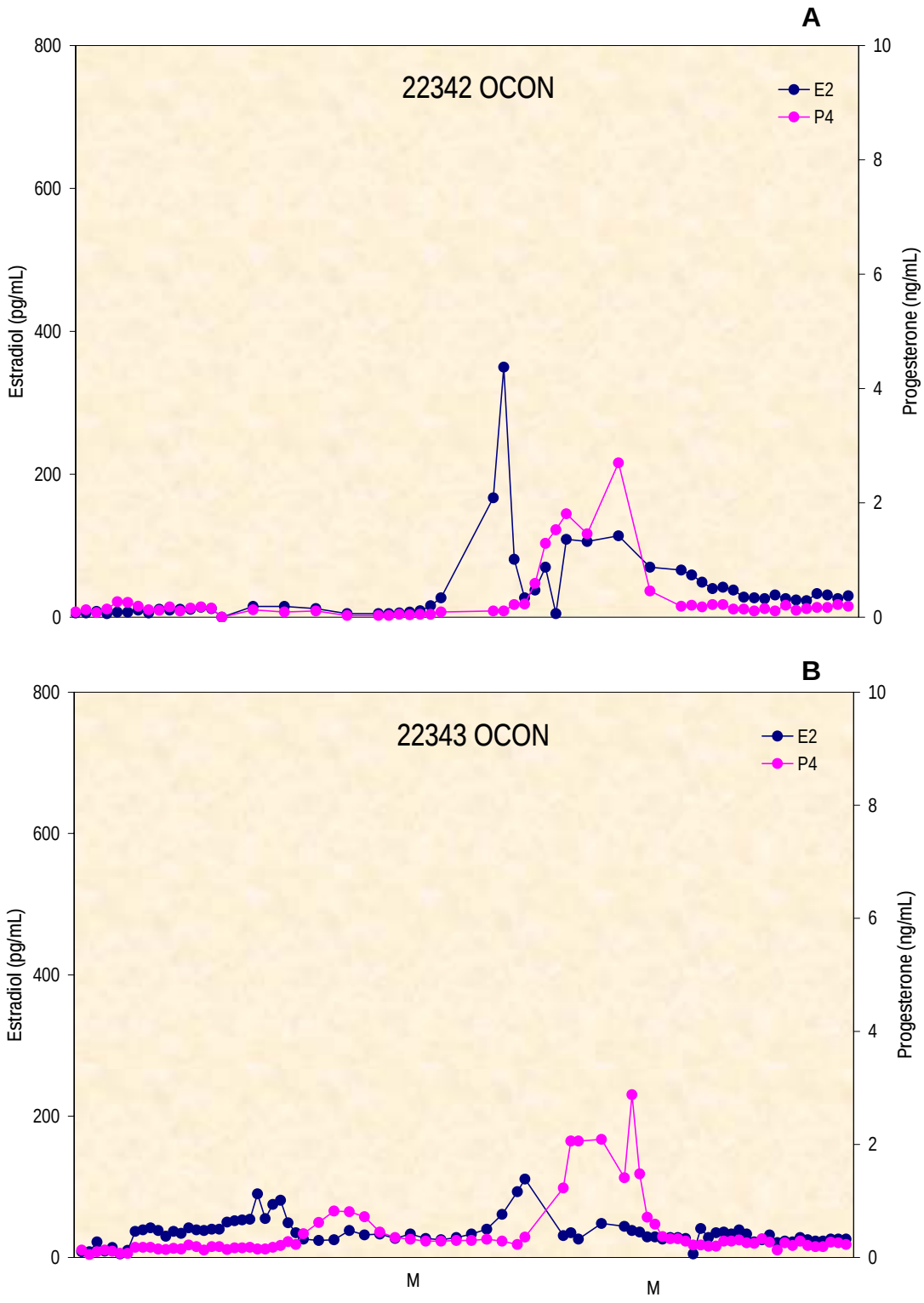

C
22352 OCON
E2
P4
Estradiol (pg/mL)
Progesterone (ng/mL)
800
600
400
200
0
10
8
6
4
2
0
M
M
M
D
22354 OCR
E2
P4
Estradiol (pg/mL)
Progesterone (ng/mL)
800
600
400
200
0
10
8
6
4
2
0
M
M
M

Figure 2.1 Short-Term (ONPRC) Regular Cyclers. Representative estradiol (E2) and progesterone (P4) graphs for 3 consecutive menstrual cycles. Profiles of regular cyclers (≥2 cycles demonstrating a mid-cycle E2 peak followed by luteal phase levels of P4) are depicted above: A) Young CON, B) Young CR, C) Old CON, D) Old CR. Onset of menses indicated by the letter "M."


A
22342 OCON
E2
P4
Estradiol (pg/mL)
Progesterone (ng/mL)
800
600
400
200
0
10
8
6
4
2
0
B
22343 OCON
E2
P4
Estradiol (pg/mL)
Progesterone (ng/mL)
800
600
400
200
0
10
8
6
4
2
0
M
M

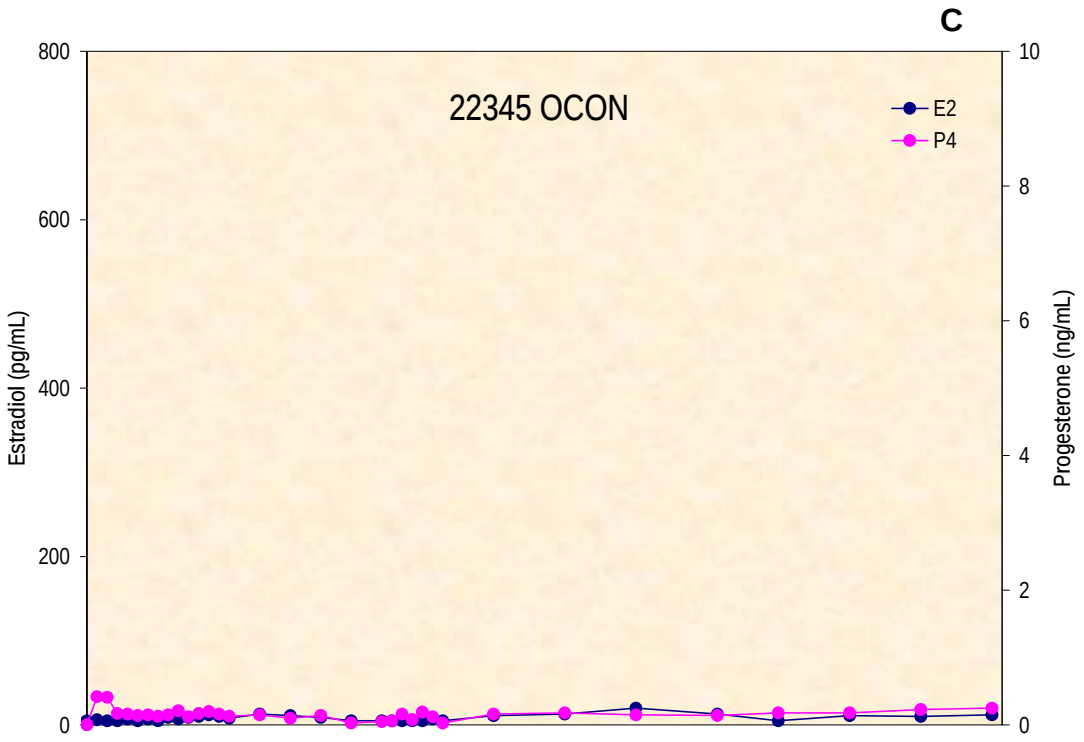


Figure 2.2 Short-Term (ONPRC) OCON Irregular Cyclers. Individual estradiol (E2) and progesterone (P4) graphs for approximately 90 days. Profiles of old CON irregular cyclers (OI-CON) are depicted above. Onset of menses indicated by "M." Three of eight OCON exhibited irregular cycles: A) 22342, B) 22343, C) 22345.

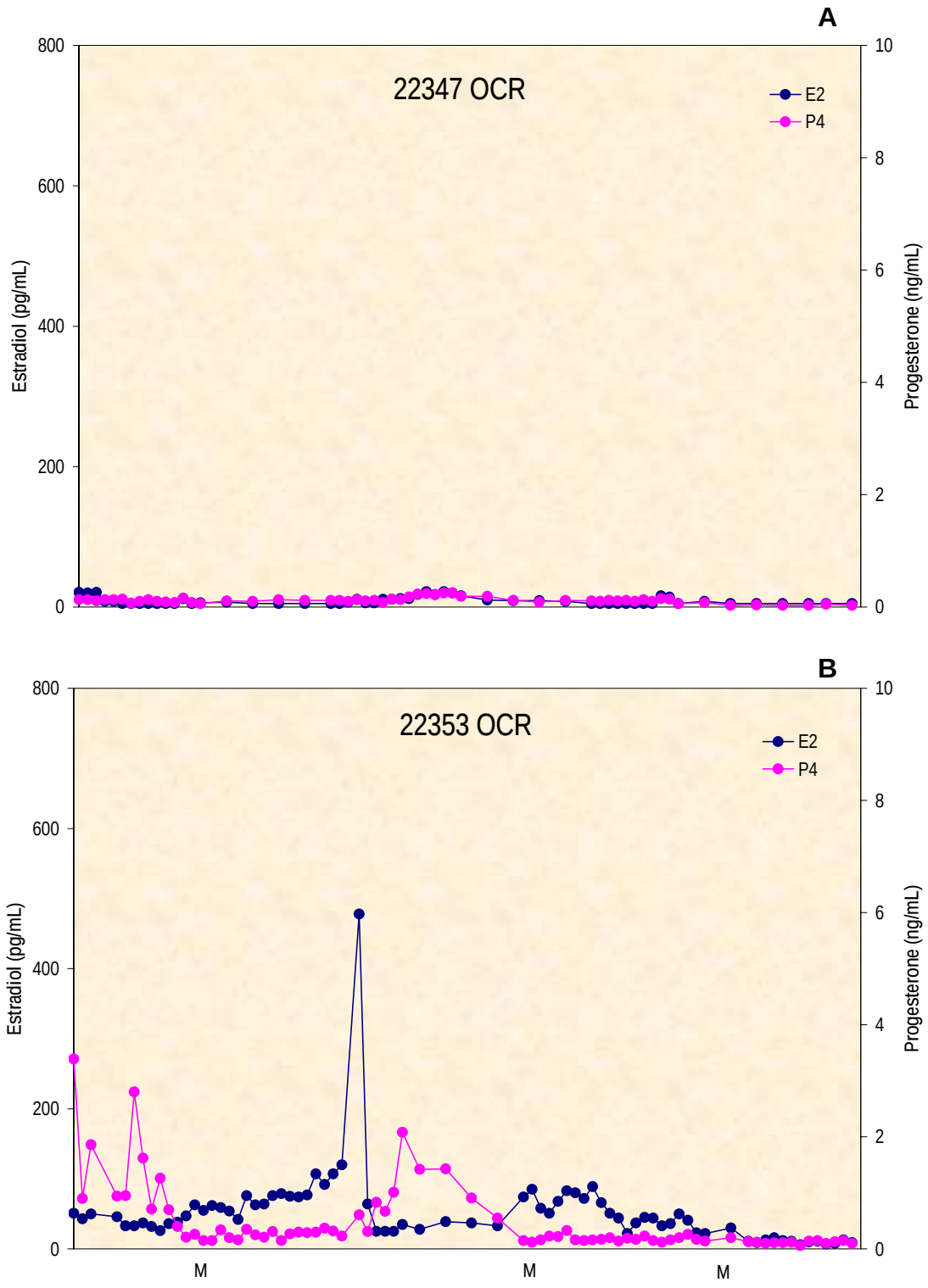
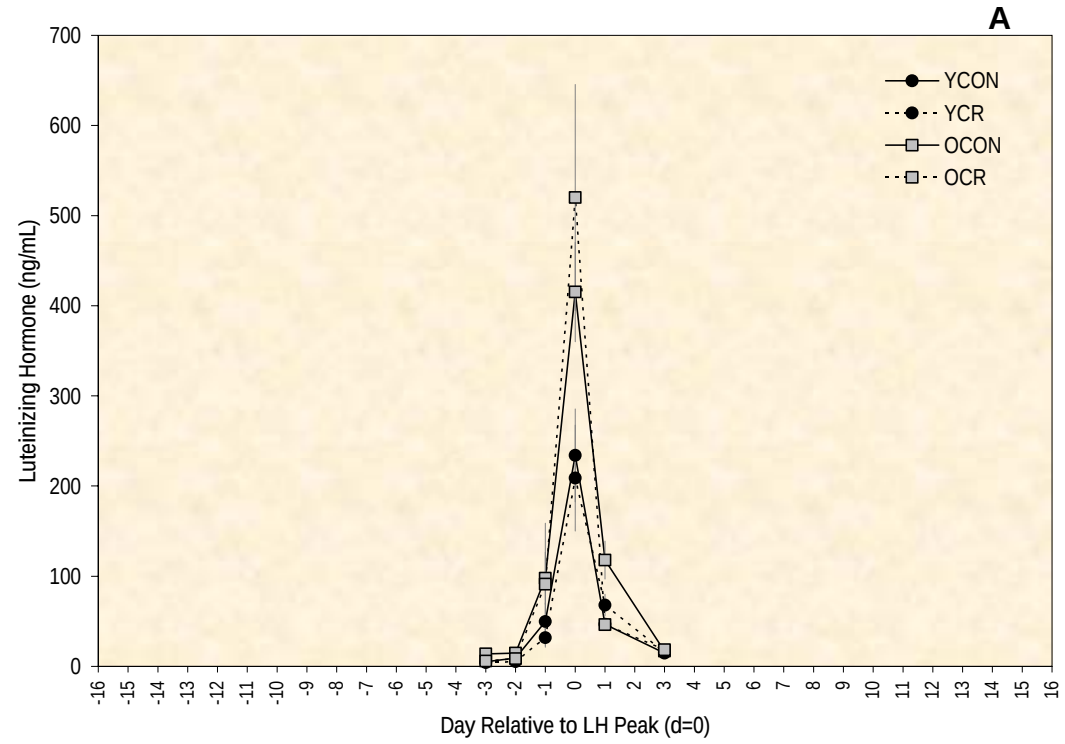
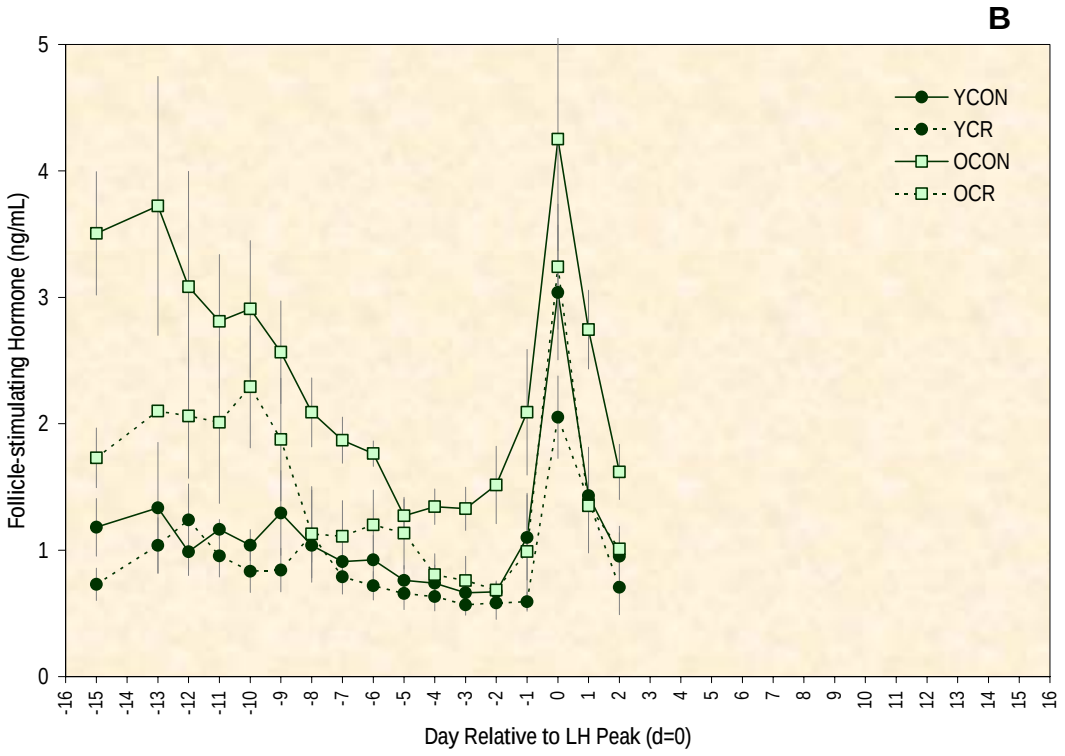
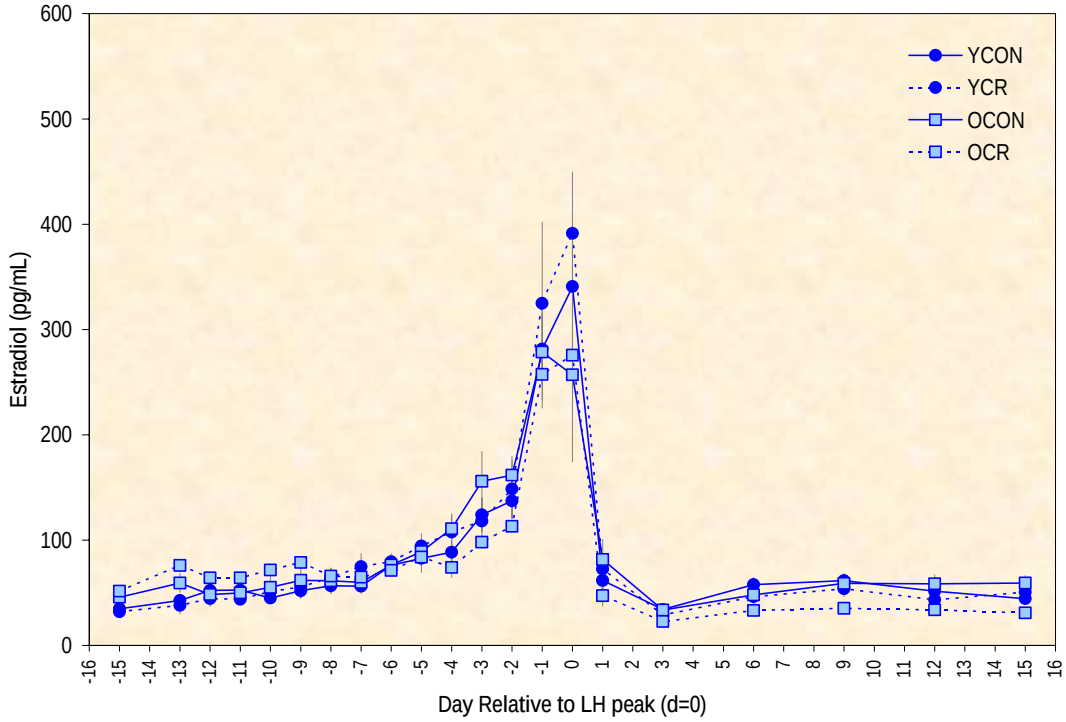


Figure 2.3 Short-Term (ONPRC) OCR Irregular Cyclers. Individual estradiol (E2) and progesterone (P4) graphs for approximately 90 days. Profiles of old CR irregular cyclers (OI-CR) are depicted above. Onset of menses indicated by "M." Two of seven OCR exhibited irregular cycles: A) 22347 and B) 22353

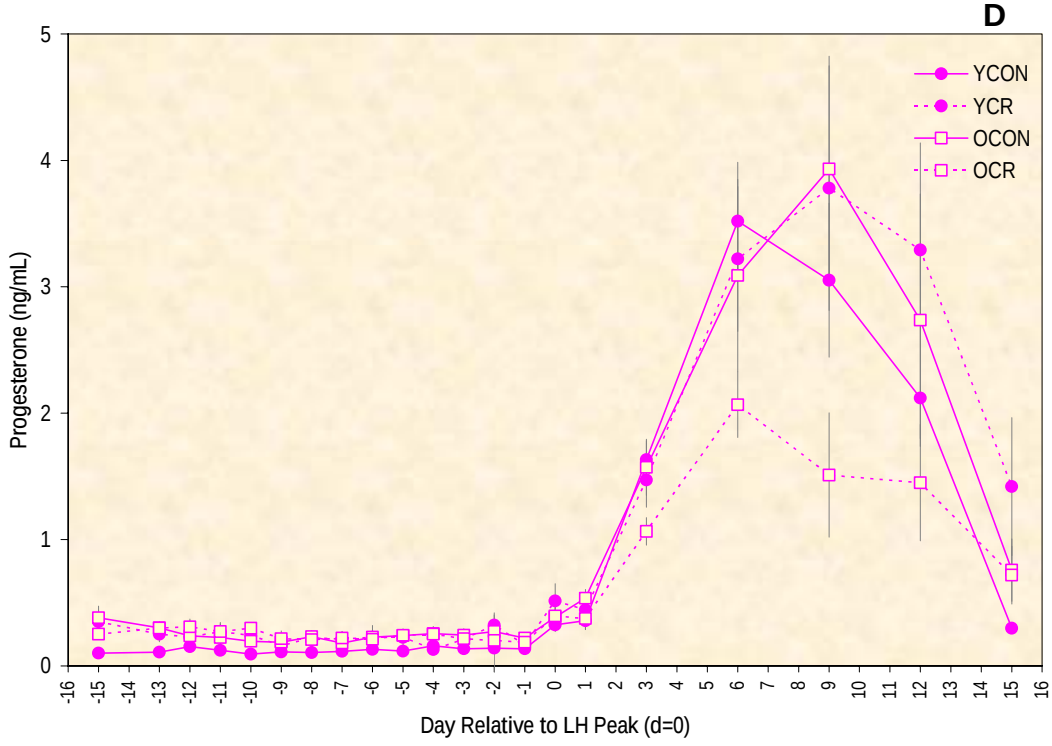

A
YCON
YCR
OCON
OCR
Luteinizing Hormone (ng/mL)
Day Relative to LH Peak (d=0)


B
YCON
YCR
OCON
OCR
Follicle-stimulating Hormone (ng/mL)
Day Relative to LH Peak (d=0)

C


YCON
YCR
OCON
OCR
Estradiol (pg/mL)
Day Relative to LH peak (d=0)

D


YCON
YCR
OCON
OCR
Progesterone (ng/mL)
Day Relative to LH Peak (d=0)

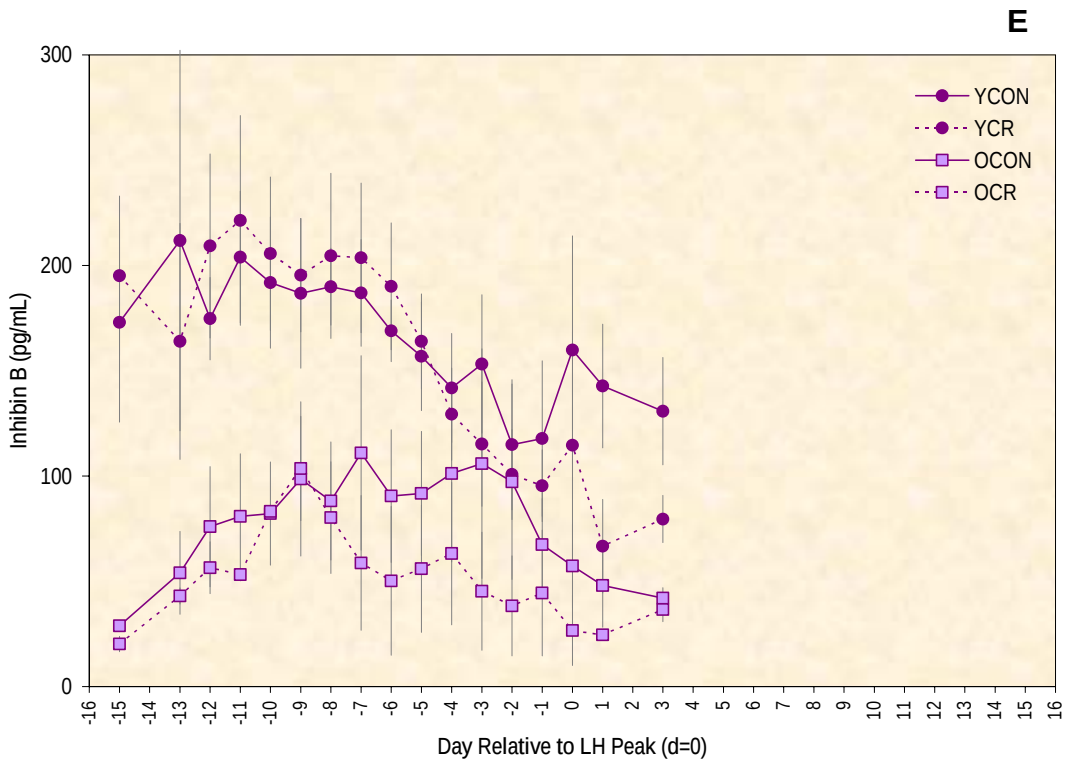


Figure 2.4 Short-Term CR (ONPRC) Hormone Profiles Relative to LH Peak. Each graph represents the hormone levels averaged for each female exhibiting regular cycles across three consecutive cycles and normalized to the estimated mid-cycle LH peak (d=0). All YCON (n=5) and YCR (n=5) were included while 6 of 8 OCON and 3 of 8 OCR were included. A) Luteinizing hormone (LH); B) follicle-stimulating hormone (FSH); C) estradiol (E2); D) progesterone (P4) and E) inhibin B (INHB).

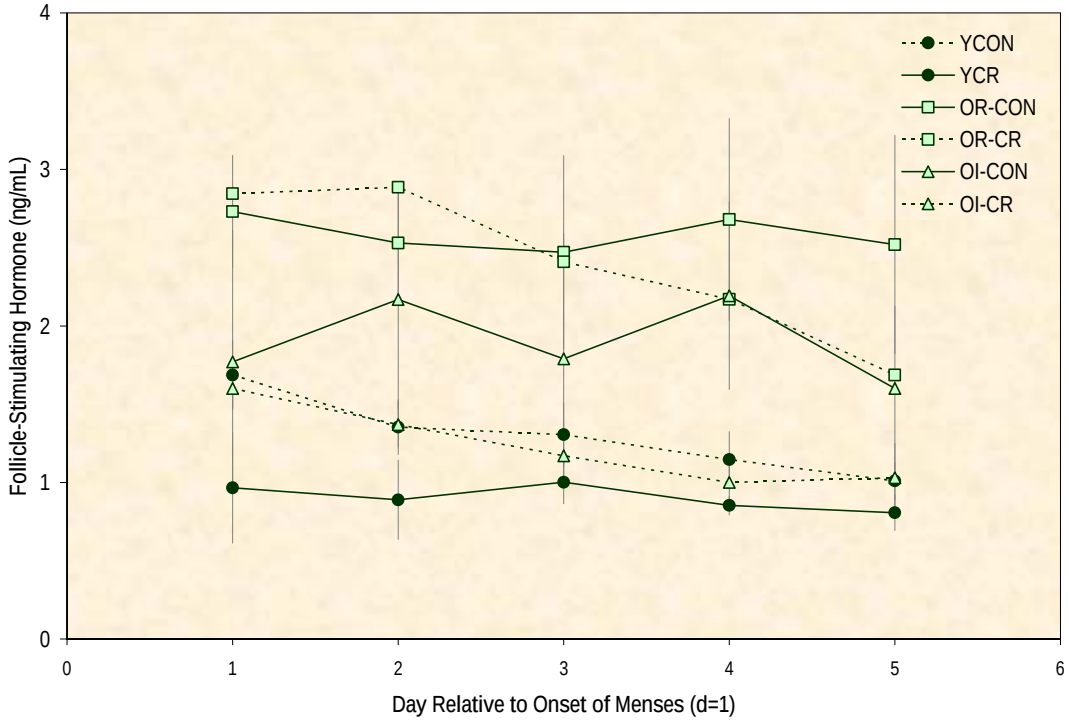


Figure 2.5 Short-Term (ONPRC) FSH Relative to Onset of Menses. Follicle-stimulating hormone (FSH) levels averaged for each female across the three consecutive cycles (or approximately 90 days) and normalized to the onset of menses or nadirs (<50pg/mL) in E2 patterns (designated d=1).

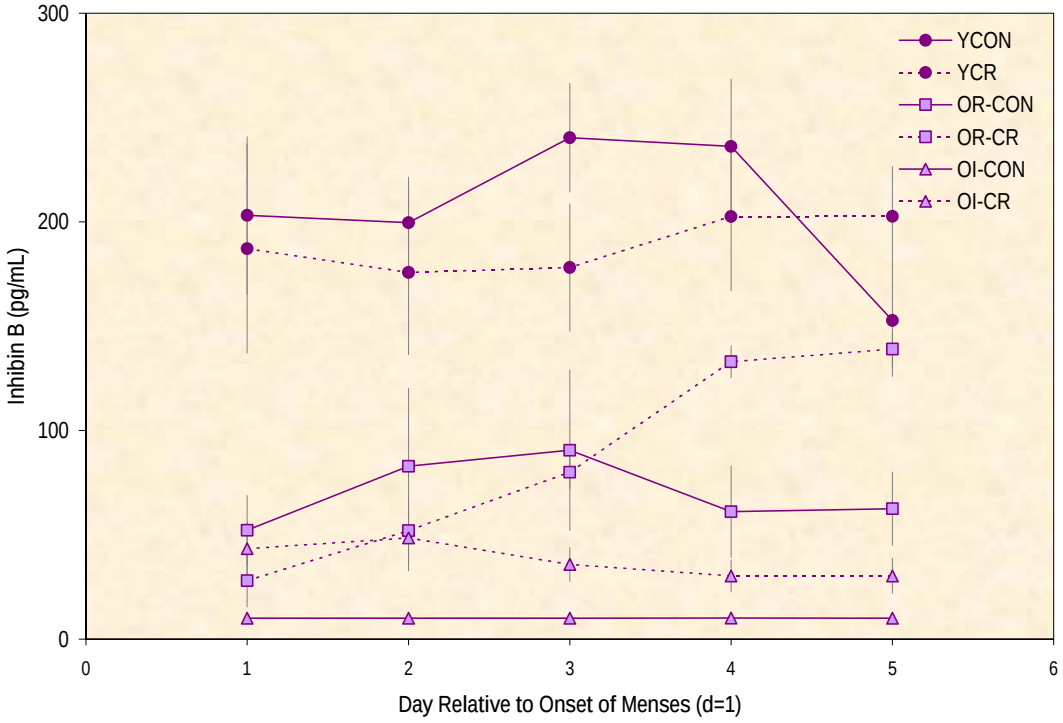
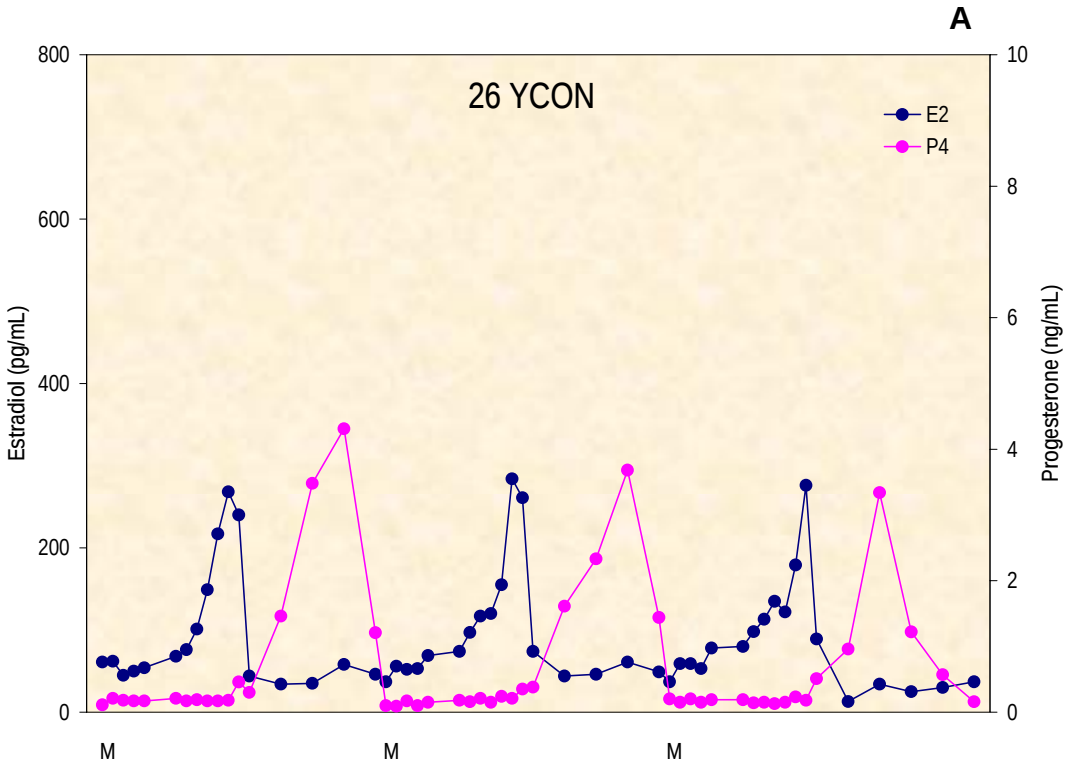
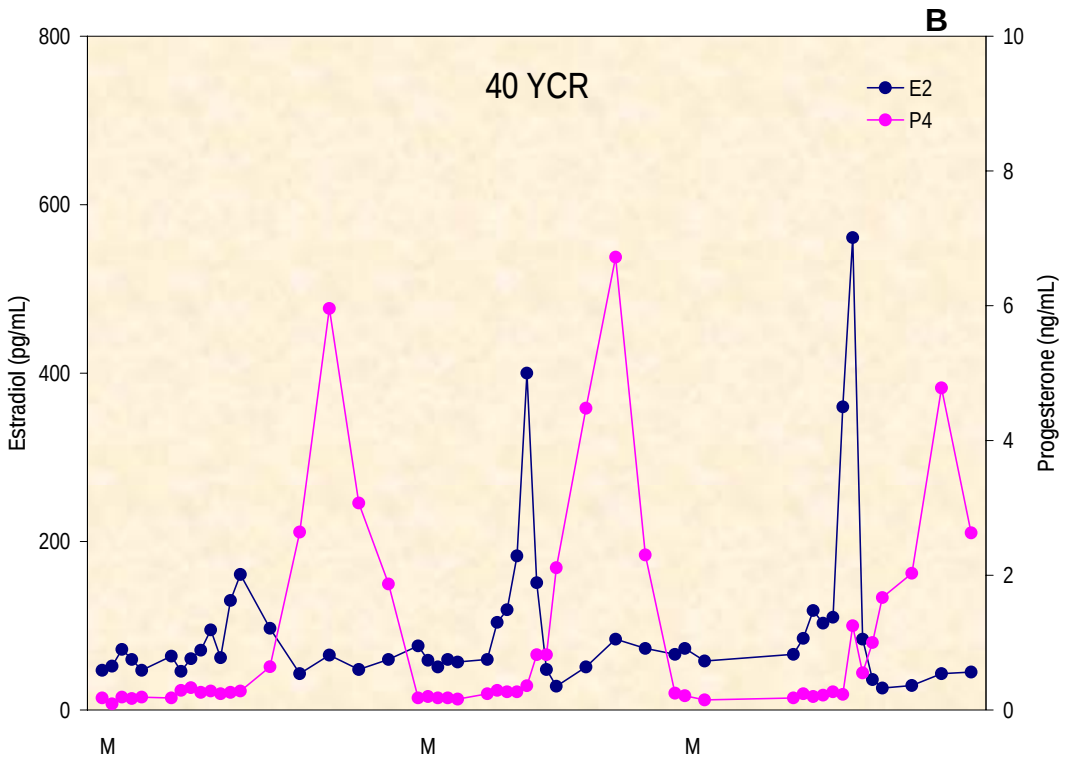


Figure 2.6 Short-Term (ONPRC) INHB Relative to Onset of Menses. Inhibin B (INHB) levels averaged for each female across the three consecutive cycles (or approximately 90 days) and normalized to the onset of menses or nadirs (<50pg/mL) in E2 patterns (designated d=1).


A
26 YCON
E2
P4
800
600
400
200
0
10
8
6
4
2
0
Estradiol (pg/mL)
Progesterone (ng/mL)
M
M
M


B
40 YCR
E2
P4
800
600
400
200
0
10
8
6
4
2
0
Estradiol (pg/mL)
Progesterone (ng/mL)
M
M
M

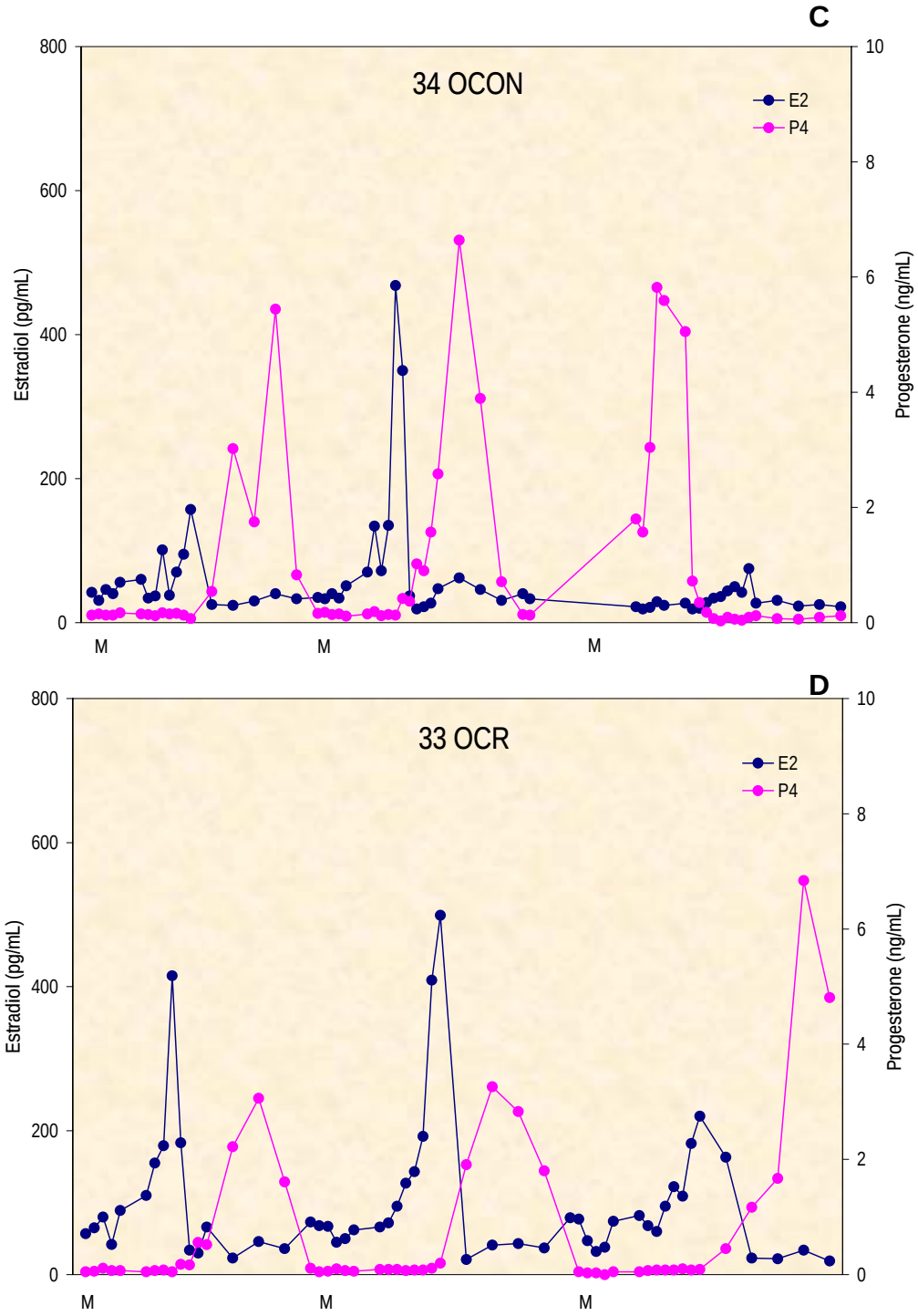
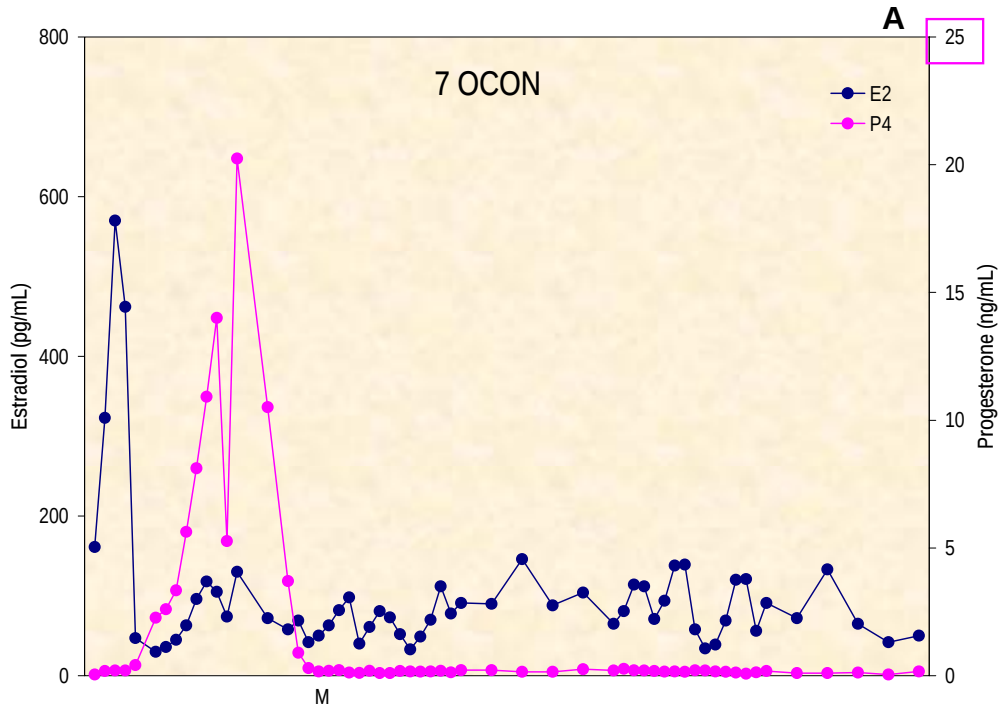
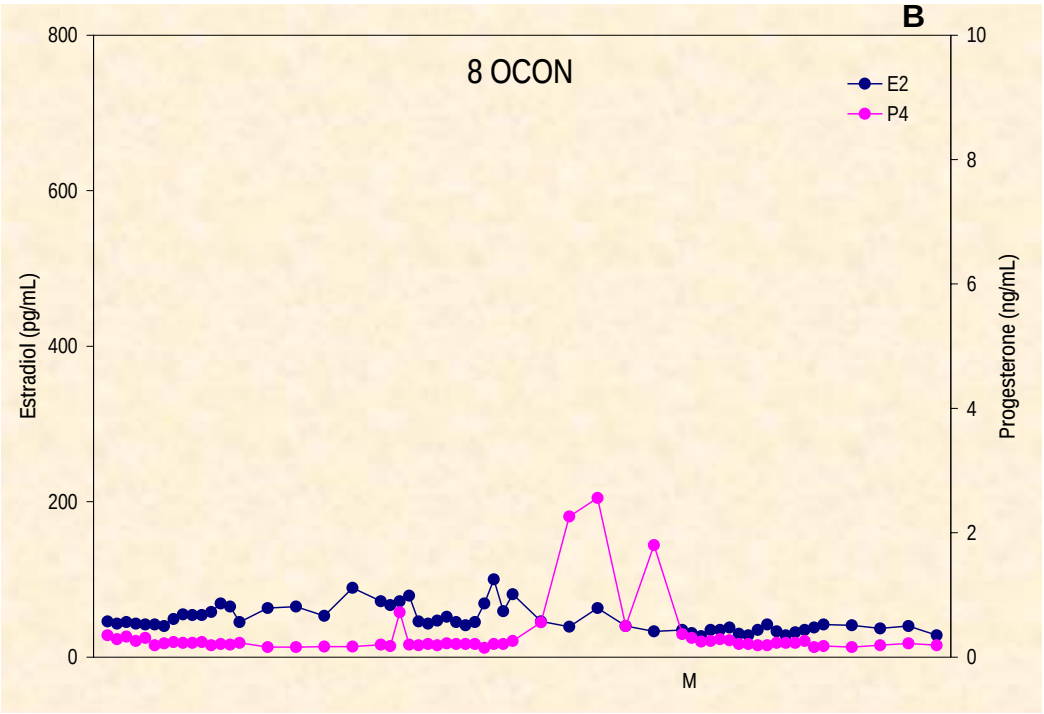


Figure 2.7 Long-Term (NIA) Regular Cyclers. Representative estradiol (E2) and progesterone (P4) profiles of regular cyclers on long-term CR (NIA). Profiles of regular cyclers (≥2 cycles demonstrating a mid-cycle E2 peak followed by luteal phase levels of P4) are depicted above: A) Young CON, B) Young CR, C) Old CON, D) Old CR. Onset of menses indicated by "M."


A
7 OCON
E2
P4
800
600
400
200
0
25
20
15
10
5
0
Estradiol (pg/mL)
Progesterone (ng/mL)
M


B
8 OCON
E2
P4
800
600
400
200
0
10
8
6
4
2
0
Estradiol (pg/mL)
Progesterone (ng/mL)
M

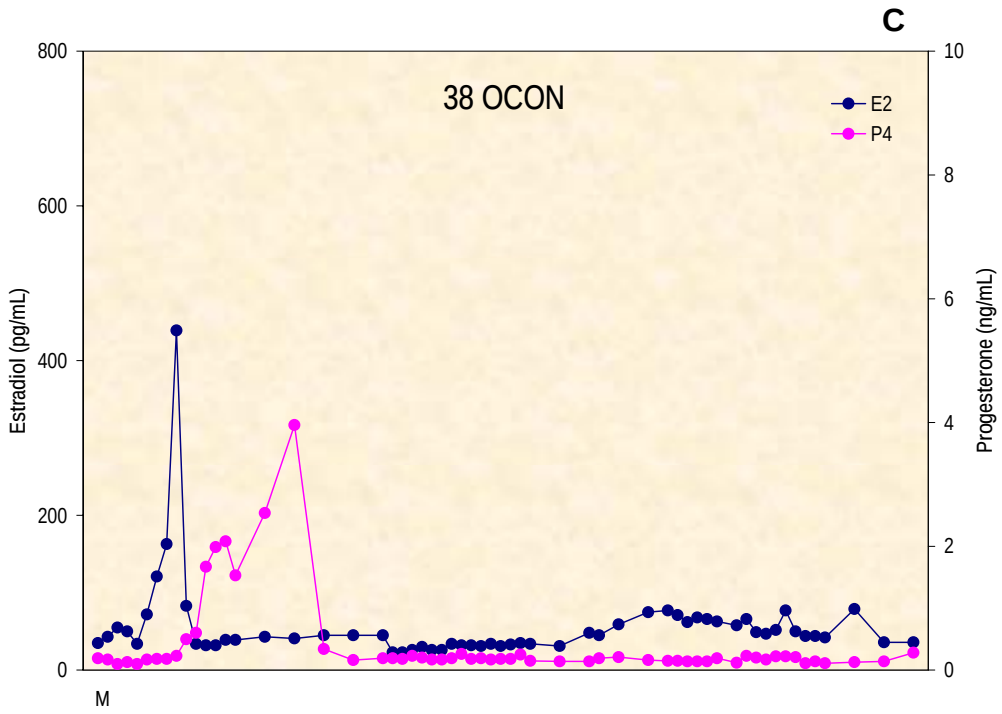
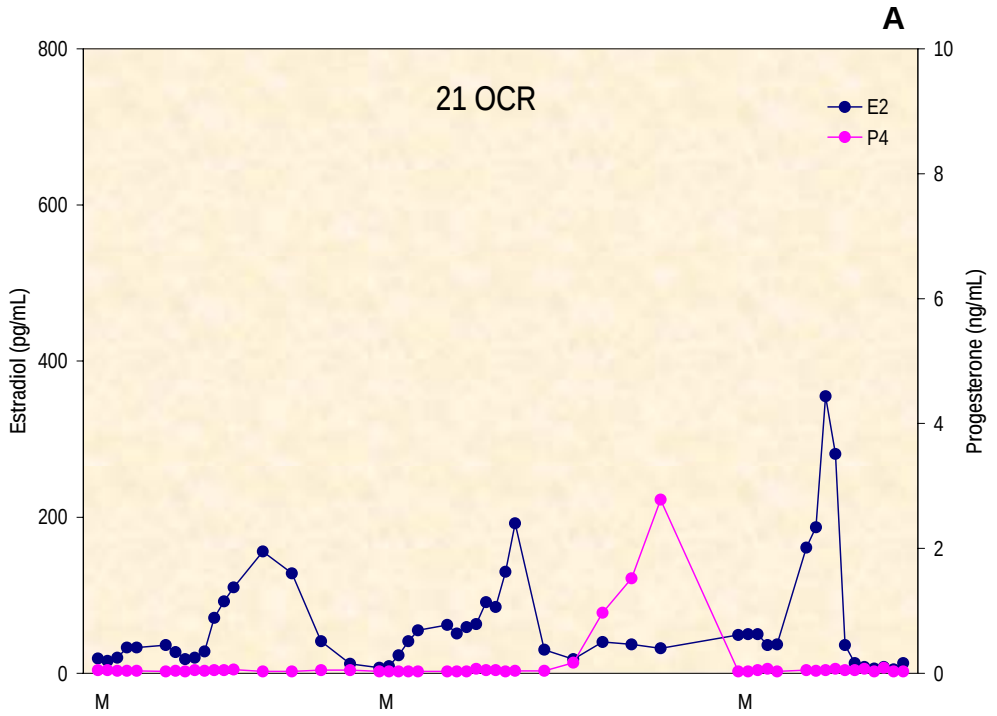
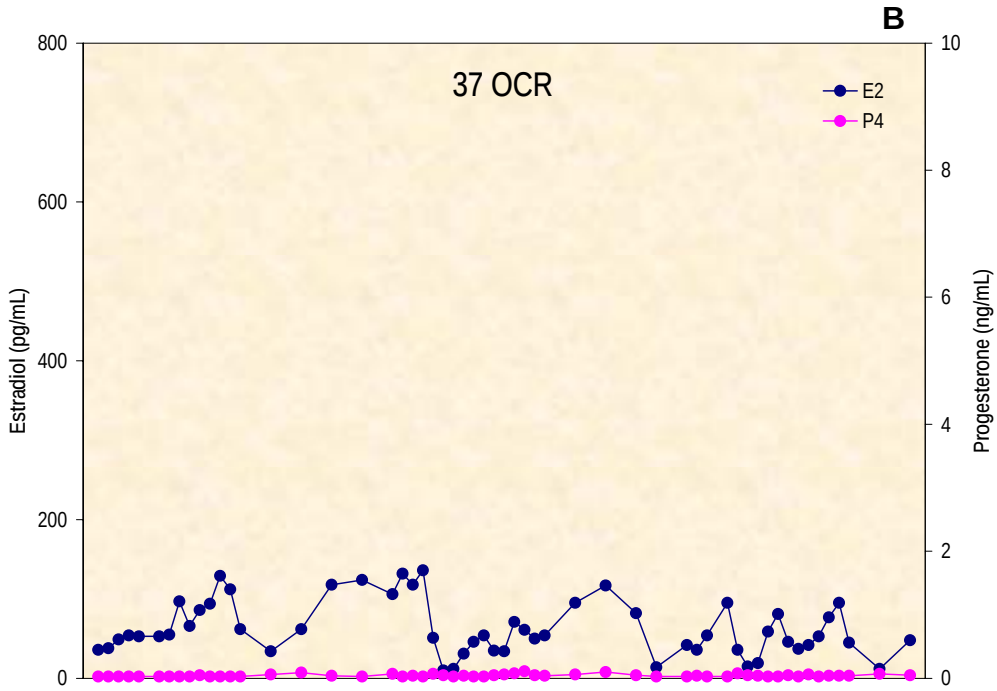


Figure 2.8 Long-Term (NIA) OCON Irregular Cyclers. Individual estradiol (E2) and progesterone (P4) graphs for approximately 90 days. Profiles of old CON irregular cyclers (OI-CON) are depicted above. Onset of menses indicated by "M." Three of six OCON exhibited irregular cycles: A) 7, B) 8, C) 38.


A
21 OCR
E2
P4
Estradiol (pg/mL)
Progesterone (ng/mL)
800
600
400
200
0
10
8
6
4
2
0
M
M
M


B
37 OCR
E2
P4
Estradiol (pg/mL)
Progesterone (ng/mL)
800
600
400
200
0
10
8
6
4
2
0

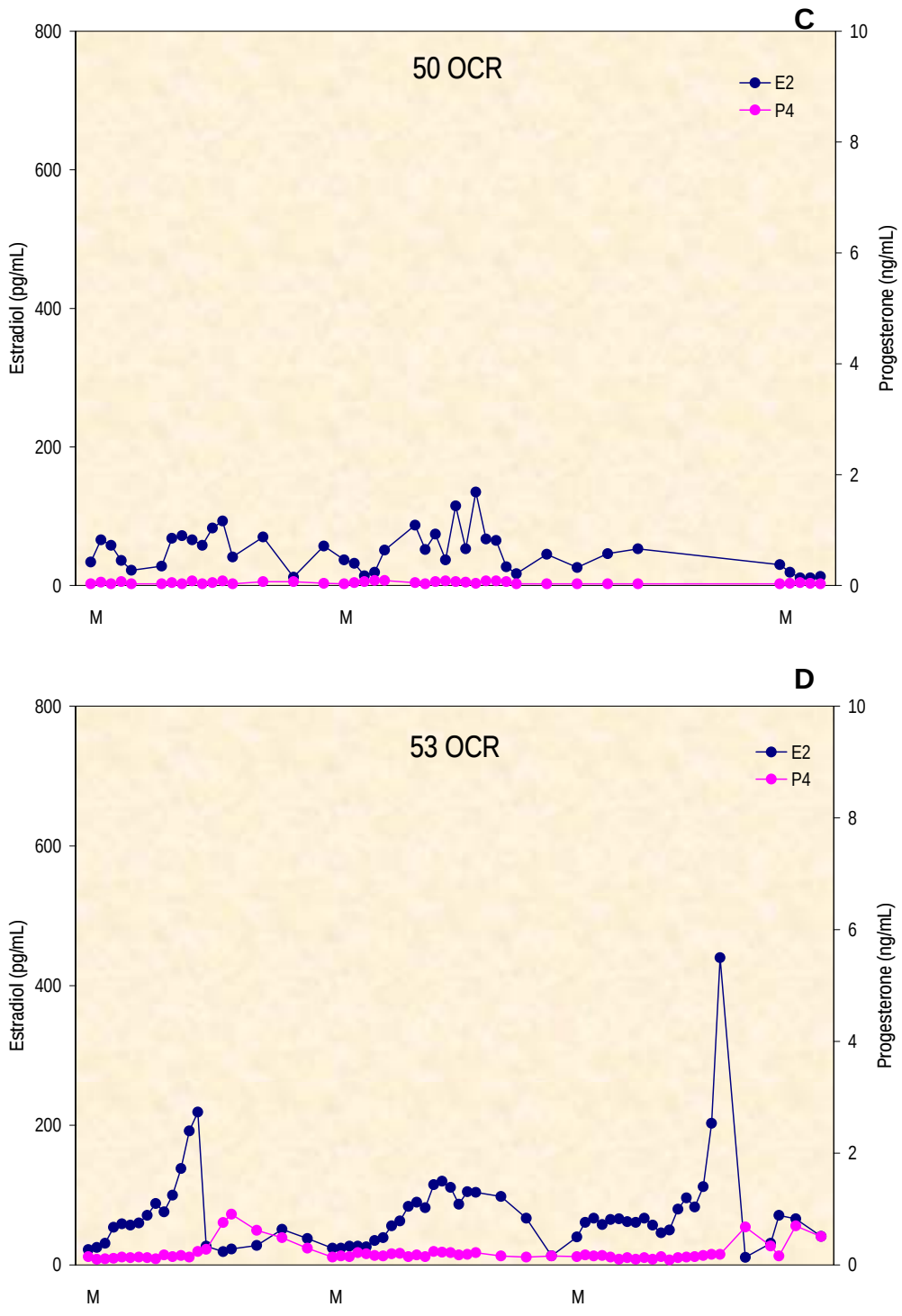
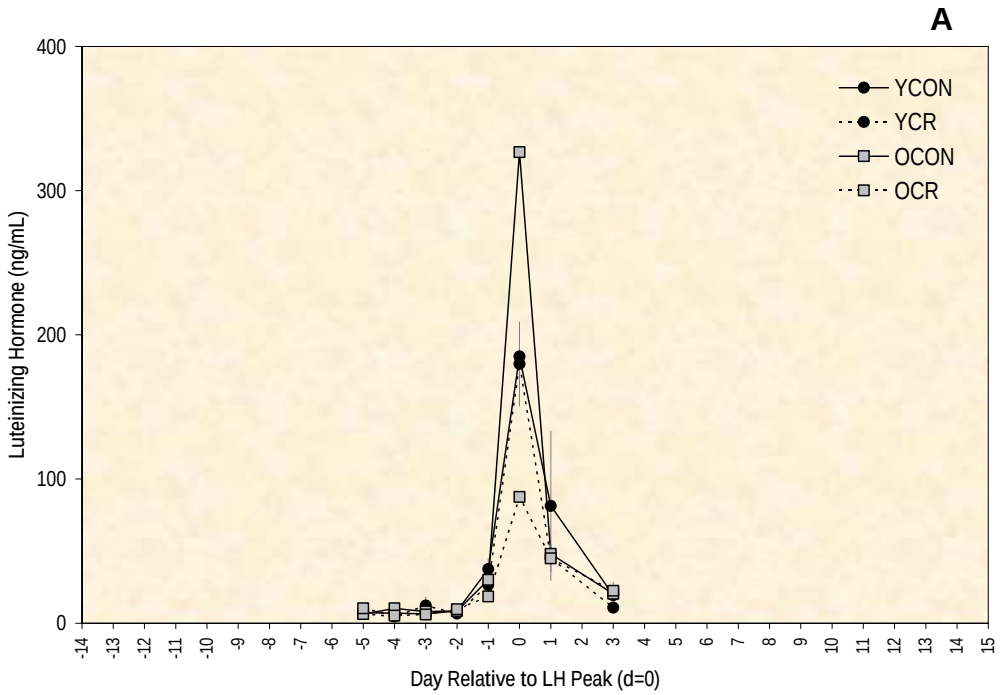
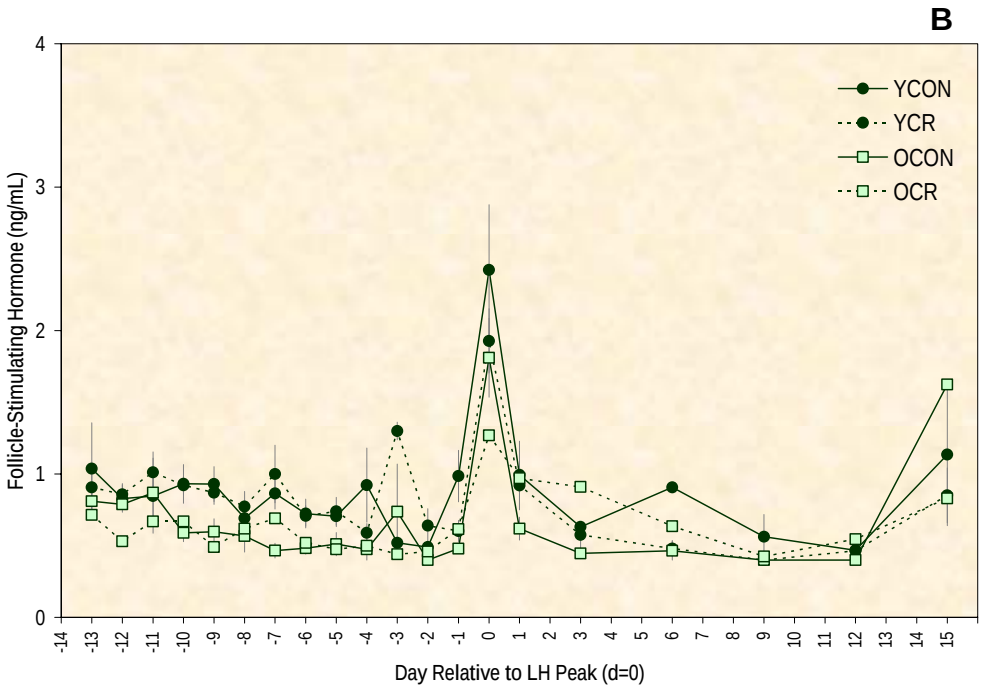
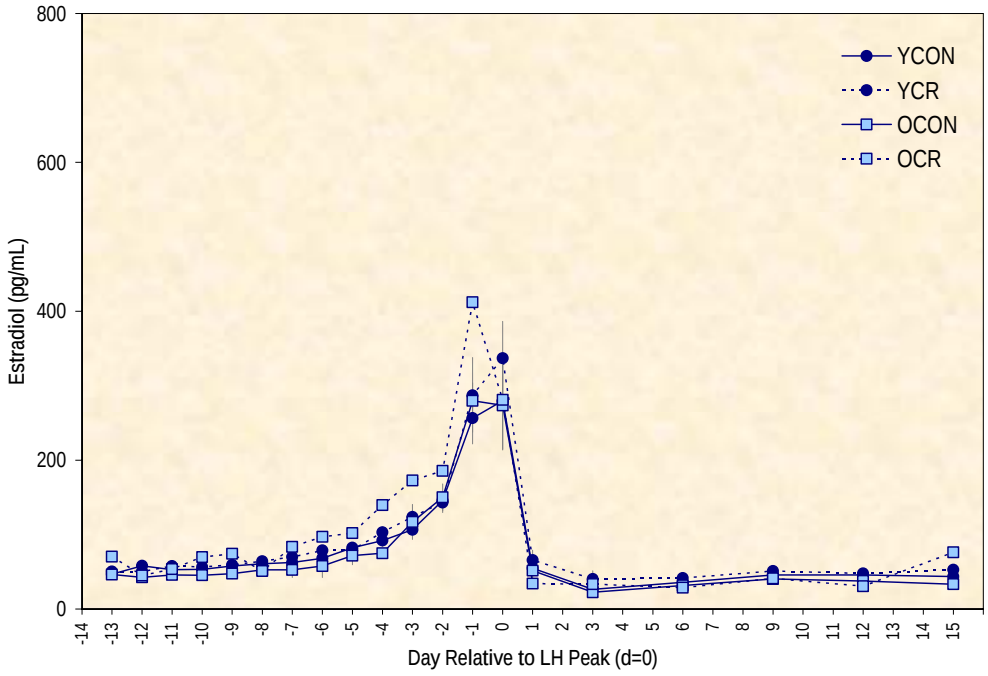


Figure 2.9 Long-Term (NIA) OCR Irregular Cyclers. Individual estradiol (E2) and progesterone (P4) graphs for approximately 90 days. Profiles of old CR irregular cyclers (OI-CR) are depicted above. Onset of menses indicated by "M." Four of five OCR exhibited irregular cycles: A) 21, B) 37, C) 50, D) 53.

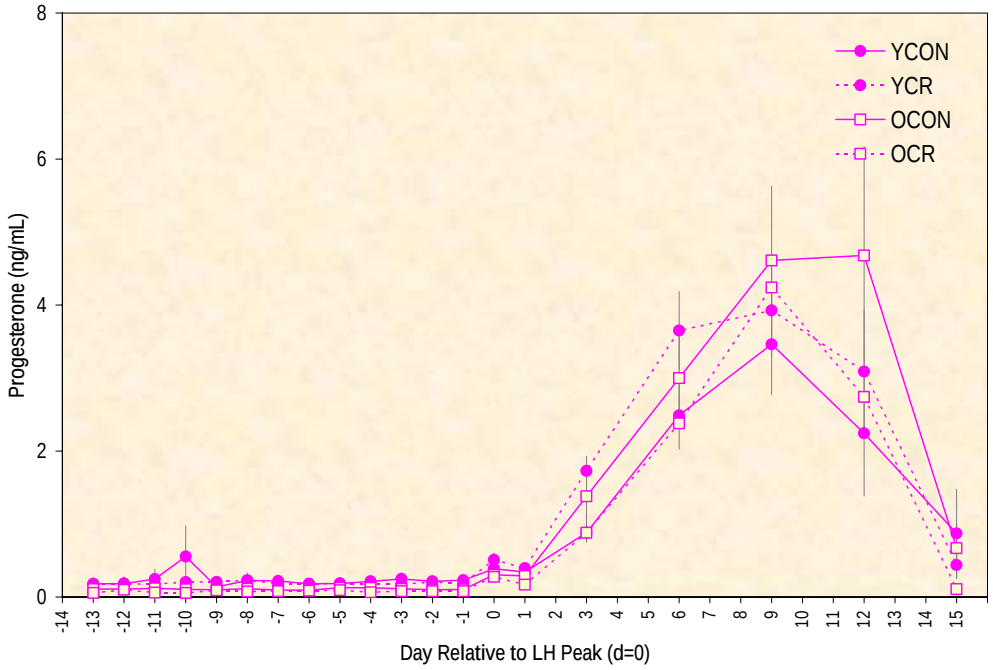

A
YCON
YCR
OCON
OCR
Luteinizing Hormone (ng/mL)
Day Relative to LH Peak (d=0)


B
YCON
YCR
OCON
OCR
Follicle-Stimulating Hormone (ng/mL)
Day Relative to LH Peak (d=0)

C


800
600
400
200
0
Estradiol (pg/mL)
YCON
YCR
OCON
OCR
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Day Relative to LH Peak (d=0)

D


8
6
4
2
0
Progesterone (ng/mL)
YCON
YCR
OCON
OCR
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5 -4 -3 -2 -1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Day Relative to LH Peak (d=0)

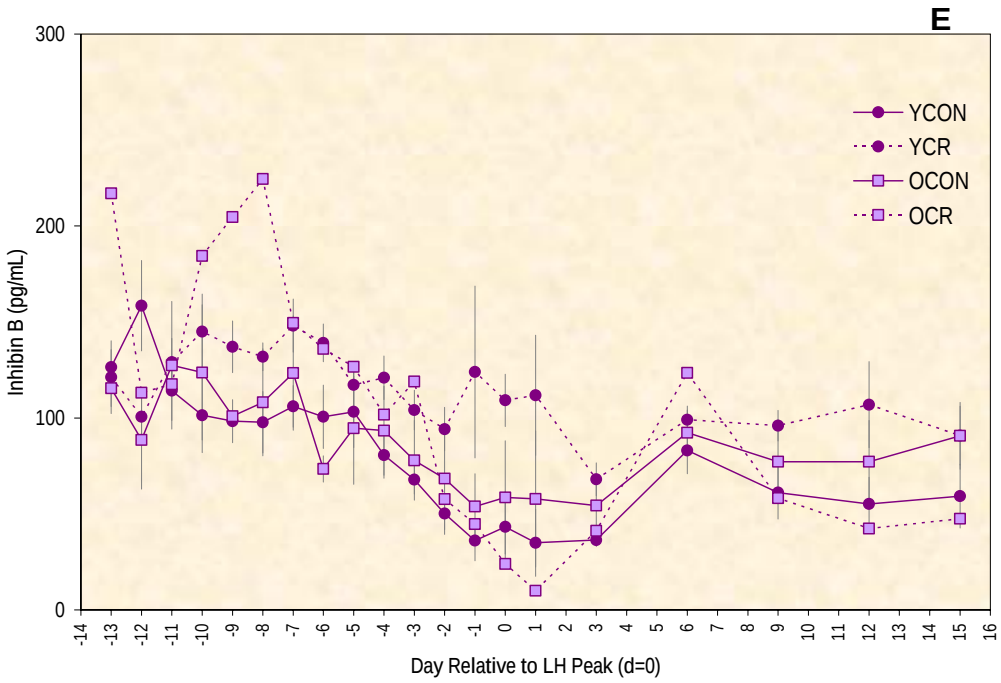


Figure 2.10 Long-Term (NIA) Hormone Profiles Relative to LH Peak. Each graph represents the hormone levels averaged for each female exhibiting regular cycles across three consecutive cycles and normalized to the estimated mid-cycle LH peak (d=0). All YCON (n=5) and YCR (n=7) were included while 3 of 6 OCON and 1 of 5 OCR were included. A) Luteinizing hormone (LH); B) follicle-stimulating hormone (FSH); C) estradiol (E2); D) progesterone (P4) and E) inhibin B (INHB).

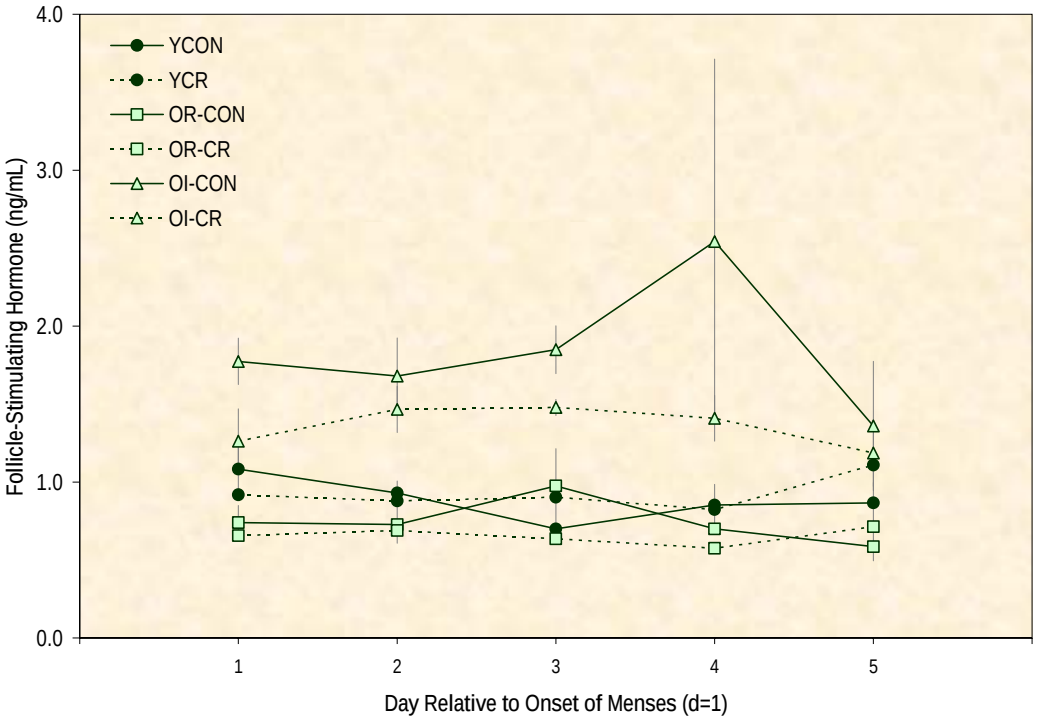


Figure 2.11 Long-Term (NIA) FSH Relative to Onset of Menses. Follicle-stimulating hormone (FSH) levels averaged for each female across the three consecutive cycles (or approximately 90 days) and normalized to the onset of menses or nadirs (<50pg/mL) in E2 patterns (designated d=1).

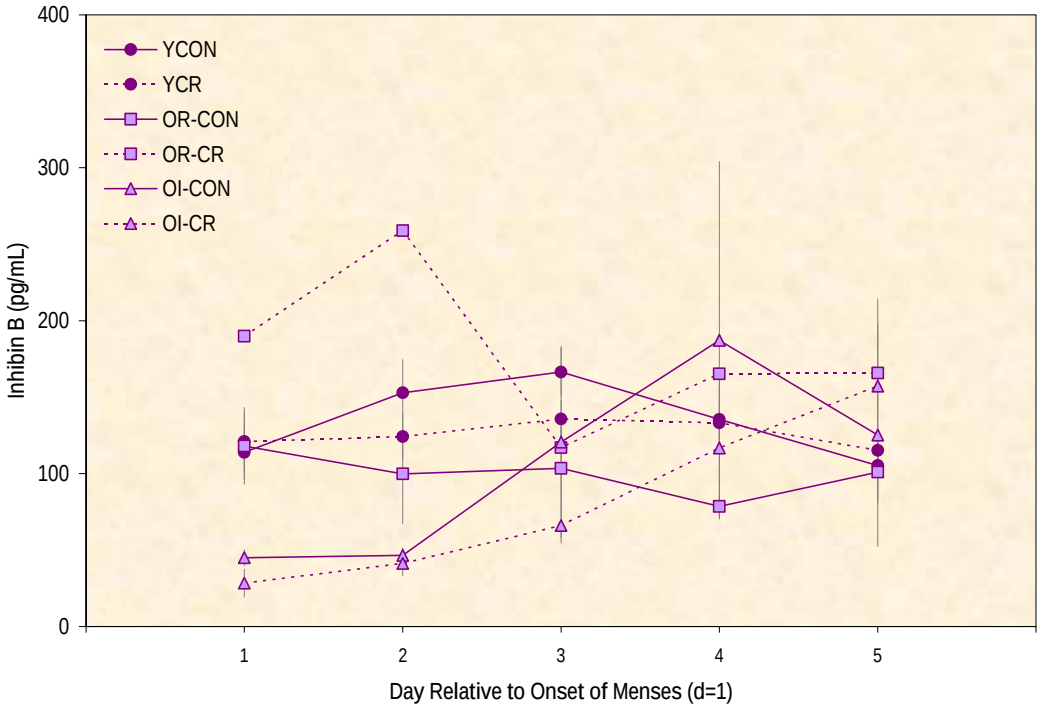


Figure 2.12 Long-Term (NIA) INHB Relative to Onset of Menses. Inhibin B (INHB) levels averaged for each female across the three consecutive cycles (or approximately 90 days) and normalized to the onset of menses or nadirs (<50pg/mL) in E2 patterns (designated d=1).

CHAPTER 3

OVARIAN RESERVE TESTS AND THEIR UTILITY IN PREDICTING RESPONSE TO CONTROLLED OVARIAN STIMULATION IN RHESUS MONKEYS

INTRODUCTION:

Considering the high price of assisted reproduction, it is considered prudent to estimate the probability of success prior to costly follicle stimulation and *in vitro* fertilization (IVF) protocols. Ovarian reserve tests (ORTs) are used to give a relative measurement of the remaining follicular pool. ORTs have become more commonplace in human clinics to assist in counseling patients with regard to chances of pregnancy success with or without exogenous hormone treatment. The monotropic rise in follicle-stimulating hormone (FSH) is often considered to be the hallmark event associated with declining fertility. Conversely, comparisons between young and peri-menopausal women have showed reduced levels of inhibin B (INHB). Of note is the fact that these hormonal changes occur prior to any measurable change in estradiol, progesterone or luteinizing hormone (Klein *et al.*, 1996).

Marked advances have been made using ORTs, in human research, with far less study using their animal model counterparts. Primarily, development of ORTs in nonhuman primate models would make it possible to identify the best animals for future research in artificial reproductive technologies. Secondarily,

controlled ovarian stimulation (COS) and *in vitro* fertilization are frequently an unavoidable alternative to natural breeding when animals are geographically isolated or are behaviorally incompatible. Finally, there is a great need to identify nonhuman primates entering menopause for basic aging research. Unfortunately, ART breeding programs can be expensive and sometimes require moderately invasive animal procedures, with no guarantee of success. Therefore, use or ORTs could assist in focusing resources on animals with the best likelihood of successful pregnancies.

Early follicular phase FSH (day 3) has been shown to correlate well with ovarian reserve. Measurement of basal FSH alone, while extremely useful, can be somewhat variable. Thus, a number of tests have been developed that when used in conjunction with basal FSH levels, have been successful in predicting ovarian response. In general, most of these tests rely on assessing hormonal response to ovarian stimulation, whether at the level of the hypothalamus, pituitary or direct action on the ovary.

Fanchin *et al.* (Fanchin et al., 1994) recently developed a supplemental protocol to the standard FSH measurements to assess ovarian reserve. The exogenous follicle stimulating hormone ovarian reserve test (EFORT) significantly improved the predictive value of basal FSH alone for IVF response. Subjects were injected with purified FSH on cycle day 3; blood was drawn prior to and 24 hours after FSH administration. Concomitant analysis of basal FSH and change in E2 following treatment was found to be effective in differentiating

between good and poor responders to IVF. Change in INHB following EFORT, has also been shown to correlate well with response to IVF (Dzik et al., 2000). Women with both low baseline INHB levels as well as reduced INHB response to EFORT were less responsive to IVF treatment.

The clomiphene citrate challenge test (CCCT) is a similar and also highly effective test of ovarian reserve. CCCT has been proven to be 93% effective in predicting infertility and was more successful in identifying low ovarian reserve patients than basal FSH alone (Kahraman et al., 1997). In young, healthy ovaries, ovarian reserve may be associated with an ample number of developing follicles. Administration of clomiphene citrate (CC), a hypothalamic estradiol receptor blocker, results in up-regulation of FSH and LH from the pituitary. Clomiphene citrate is an orally active non-steroidal chemical that is a mixture of two isomers: zuclomiphene and euclomphene; zuclomiphene being more active of the two (Speroff et al., 1999). Clomiphene itself, is weakly estrogenic; however, due to its ability to bind receptor for long periods of time, results in reduced negative feedback and increased gonadotropins, likely via activation of GnRH secretion. This stimulates follicular growth and subsequent production of inhibin and estradiol (Speroff et al., 1999). In the case of a peri-menopausal ovary, reduced follicle number results in a reduced inhibin response and presumably reduced negative feedback at the level of the pituitary and/or hypothalamus. In human clinical trials, the CCCT is commonly used in conjunction with day 3 (with onset of mense as day 1 of the menstrual cycle)

FSH levels to predict relative ovarian reserve. Patients are hormonally monitored to determine cyclicity, using P4 and LH. Basal FSH and E2 are measured in early follicular phase (days 2-5) and 100 mg clomiphene citrate is administered orally once daily in mid follicular phase (days 5-9). FSH and E2 are measured in late follicular phase (days 9-11). The results of the basal versus clomiphene citrate-stimulated FSH and E2 may be used to predict relative IVF success. Cessation of CC results in suppression of FSH, likely via endogenous ovarian inhibins. INHB may be measured in combination with the CCCT as a direct measure of ovarian responsiveness. It has been shown that women with normal ovarian reserve have higher granulosa cell INHB production as compared with women with diminished ovarian reserve (Hofmann et al., 1998). Thus, the results of the CCCT are evaluated considering that younger women will a large ovarian reserve respond to CCCT with a dramatic change in estradiol, comparing basal to stimulated hormone levels. Older women with significantly reduced ovarian reserve respond to CCCT with little to no change in estradiol, when comparing basal to stimulated hormone levels.

The first detectable hormone changes are measurable in women years before the final climacteric (Robertson and Burger, 2002; Santoro, 2005). Thus the potential for a menopause delaying regimen would be extremely beneficial. In rats, moderate calorie restriction (CR) has been shown to be an effective method of delaying the onset of ovarian decline (Holehan and Merry, 1985c; Merry and Holehan, 1979). Results were somewhat mixed in mice maintained on moderate CR (with regard to onset of puberty), although delayed ovarian

decline was confirmed (Nelson et al., 1985). Preliminary results in rhesus monkeys suggest that animals continue to cycle normally (Wu et al., 2004), however, further research must still be performed.

The objective of this study was two-fold. We evaluated several commonly used clinical ORTs and hormone indices, to predict response to controlled ovarian stimulation in rhesus monkeys. These included: day 3 (d3) follicle-stimulating hormone (FSH), day 3 inhibin B (INHB); the clomiphene citrate challenge test (CCCT); and the exogenous follicle-stimulating hormone test (EFORT). Additionally, we used those ORTs to assess the effect of moderate CR in these animals. This study is the first to evaluate the use of ORTs in nonhuman primates and offers valuable information to both clinical, laboratory and captive settings alike.

Materials and Methods

Animals and Diet

Female rhesus monkeys (*Macaca mulatta*) were identified for this study and maintained on a control diet *ad libitum* to determine average daily feed intake (AFI). AFI was then divided into two meals and control (CON) monkeys received the full amount, while calorie restricted (CR) monkeys were reduced by 10% each month until the desired 30% CR was achieved. Young (Y; 12 years) and old (O; 19-26 years) monkeys were distributed as follows: YCON, n=4; YCR, n=4; OCON, n=9; OCR, n=5.

The diet has been described in detail by Lane *et al.*, 2001 and represents restricted intake of a nutritionally replete diet. A 30% calorie restriction is considered moderate, and the animals appear to continue to cycle normally.

Ovarian Reserve Tests

All monkeys were monitored for onset of menses (d=1), and several clinically used ovarian reserve tests (ORTs) were evaluated for diagnostic accuracy in predicting response to controlled ovarian stimulation (COS) in rhesus monkeys. The ORTs included: day 3 follicle-stimulation hormone (FSH), day 3 inhibin B (INHB), the clomiphene citrate challenge test (CCCT) and the exogenous FSH ovarian reserve test (EFORT). ORT predictions were retrospectively compared to a controlled ovarian stimulation (COS) to determine prediction accuracy.

Blood samples were collected from the saphenous vein in trained monkeys with no anesthesia. Serum was then stored at -20˚C until assayed. Samples were assayed for estradiol (E2) and FSH at the Oregon National Primate Research Center (ONPRC) Endocrine CORE.

Inhibin B was determined using the commercially available Active Inhibin B kit (Diagnostic Systems Laboratory; Webster, TX). This ELISA was validated for use with rhesus monkey serum in our laboratory. Parallel displacement curves were obtained by comparing serially diluted (1:1 to 1:16) pooled rhesus monkey serum against known INHB concentrations. Recovery of known concentrations of unlabeled INHB added to pooled rhesus monkey serum was

100% with a coefficient of variation of 10%. Inter-assay coefficients of variation for two internal controls were 15% and 9%. The intra-assay coefficient of variation was always less than 10%.

Ovarian Reserve Tests

Mean ± SE hormone levels were similar between diet groups within ages and COS outcome. Therefore, subsequent analyses grouped CON and CR. ORTs were evaluated based on individual hormone responses and COS outcome was retrospectively used to classify OCON and OCR into successful or unsuccessful COS (COS+/-). Chi-square analyses were then performed to evaluate ORT predictions relative to actual COS outcomes.

Day 3 FSH

During a spontaneous menstrual cycle, serum FSH was determined on day 3 (d3; menses=d1). Based on human clinical data, elevated early follicular phase FSH has been shown to indicate reduced ovarian reserve and consequently reduced COS response. D3 FSH values were used to predict COS outcome (successful, COS+ and unsuccessful, COS-) as follows:

Table 3.1 D3 FSH Criteria for Predicting COS Outcome. Criteria for prediction outcome following controlled ovarian stimulation (COS). Day 3 follicle-stimulating hormone (FSH; menses = day 1) less than or equal to 1.0 ng/mL predicts a successful COS (COS+); Day 3 FSH greater than 1.0 ng/mL predicts an unsuccessful COS (COS-).

Hormone	Criteria	Prediction
d3 FSH	≤ 1.0 ng/mL	COS+
	> 1.0 ng/mL	COS-

Day 3 INHB

During a spontaneous menstrual cycle, serum INHB was determined on d3. Inhibin B levels are inversely related to FSH levels and reduced INHB levels have been shown to indicate poor ovarian reserve and reduced COS response. D3 INHB levels were used to predict COS outcome as follows:

Table 3.2 D3 INHB Criteria for Predicting COS Outcome. Criteria for prediction outcome following controlled ovarian stimulation (COS). Day 3 inhibin B (INHB; menses = day 1) greater than or equal to 50 pg/mL predicts a successful COS (COS+); d3 INHB less than 1.0 ng/mL predicts an unsuccessful COS (COS-).

Hormone	Criteria	Prediction
d3 INHB	≥ 50 pg/mL	COS+
	< 50 pg/mL	COS-

CCCT

During a spontaneous menstrual cycle, 50 mg clomiphene citrate was administered orally on d5-d9 and serum FSH, E2 and INHB were determined on d3 and d10 (Figure 1). Clomiphene citrate is believed to act by blocking estrogen receptors at the level of the hypothalamus and/or pituitary. Reduced feedback at these tissue sites results in the stimulation of gonadotropins and increased stimulation at the level of the ovary. In a young female, the ovary contains a large cohort of developing follicles; therefore, it will respond to increased levels of gonadotropins with an amplified production of ovarian hormones. Consequently, gonadotropin production would perceive higher levels of ovarian hormones and inhibit production of gonadotropins. Therefore, clinicians expect to see increased levels of E2 and reduced levels of FSH in women with a large ovarian reserve. Conversely, a woman with poor ovarian reserve would respond with low levels of

E2 and high or unchanged levels of FSH. Standard CCCT predicts COS outcome based on d3 and d10 FSH changes alone. We evaluated INHB and E2 changes as well. CCCT hormone changes were used to predict COS outcome as follows:

Table 3.3 CCCT Criteria for Predicting COS Outcome. Criteria for prediction outcome using the clomiphene citrate challenge test (CCCT) following controlled ovarian stimulation (COS) using either follicle-stimulating hormone (FSH) or inhibin B (INHB) alone; or in combination with fold change in Estradiol (E2).

Hormone 1	Criteria 1	Hormone 2	Criteria 2	Prediction
d3 and d10 FSH	≤ 1.0			COS+
d3 and/or d10 FSH	> 1.0			COS-
d3 FSH	> 1.0	d10 E2	> 2 fold	COS+
	> 1.0	d10 E2	> 2 fold	COS-
d3 and d10 INHB	≥ 50 pg/mL			COS+
d3 and/or d10 INHB	< 50 pg/mL			COS-
d3 INHB	≥ 50 pg/mL	d10 E2	> 2 fold	COS+
	< 50 pg/mL	d10 E2	> 2 fold	COS-

EFORT

During a spontaneous menstrual cycle, Antide (Ares Serono; 0.5 mg/kg BW subcutaneous, at 0800) and recombinant human FSH (r-hFSH; Gonal-F; Ares Serono; 30 IU; intramuscular at 0800 and 1700) was administered. Whole blood was collected from the saphenous vein just prior to (d1) and approximately 24 hours following (d2) the first injection of r-hFSH. Fold change in E2, FSH and INHB was used to predict COS outcome and diagnostic accuracy was determined based on true COS response.

Table 3.4 EFORT Criteria for Predicting COS Outcome. Exogenous Follicle-Stimulating Hormone Ovarian Reserve Test (EFORT) criteria for predicting COS outcome using fold change in Estradiol (E2), Follicle-Stimulating Hormone (FSH) or Inhibin B (INHB).

Hormone	Criteria	Prediction
d2 E2	≥ 1.5 fold change in d1 E2	COS+
	< 1.5 fold change in d1 E2	COS-
d2 FSH	≥ 1.5 fold change in d1 FSH	COS+
	< 1.5 fold change in d1 FSH	COS-
d2 INHB	≥ 1.5 fold change in d1 INHB	COS+
	< 1.5 fold change in d1 INHB	COS-

COS

In a spontaneous menstrual cycle, Antide (Ares Serono; 0.5 mg/kg BW, sc, at 0800h) was administered on d1-d9 to inhibit endogenous gonadotropin production and recombinant human FSH (r-hFSH; (Gonal-F; Ares Serono; 30 IU intramuscularly at 0800 and 1700) was administered on d1-d6 followed by r-hFSH + r-h luteinizing hormone (Lahdi; Ares Serono; im, at 0800) on d6-d9 to stimulate the production of multiple preovulatory follicles. This was followed by an injection of h-chorionic gonadotropin (Serono; 1000 IU, im) on d10 or d11 to induce ovulatory maturation. Trans-abdominal ultrasonography was performed on d7 to confirm the presence of a minimum of 3 follicles of 4mm diameter on each ovary. Blood samples were also collected daily from the saphenous vein and E2 levels were determined to indirectly assess follicular response. As part of a separate experiment, follicle aspiration via laparoscopy was performed 27 h following the r-hCG injection. COS was classified as either successful (COS+) or unsuccessful (COS-) based on the following criteria:

Table 3.5 COS Criteria for Outcome. Controlled Ovarian Stimulation (COS) criteria for classifying a successful (COS+) or unsuccessful (COS-) response.

Hormone	Criteria	Ultrasound Criteria	Outcome
peak E2	> 1000 pg/mL	> 3 follicles of 4 mm diameter / ovary	COS+
	< 600 pg/mL	< 3 follicles of 4 mm diameter / ovary	COS-

Hormone Assays:

Monkeys were monitored for onset of menses (designated Menses d1). Serum was collected from the saphenous vein for 3 cycles (or approximately 90 days) following a spontaneous menses: daily during the follicular phase and every third day during the luteal phase. Circulating hormones were assayed for estradiol (E2) by specific electrochemoluminescent assay using a Roche Elecsys 2010 assay instrument at the Endocrine Services Laboratory Core, ONPRC (Beaverton, OR). Follicle-stimulating hormone (FSH) was determined using an in-house radioimmunoassay (RIA). Hormone concentrations were validated against previous RIAs in this laboratory (Hess et al., 1981; Zelinski-Wooten et al., 1995).

Inhibin B was determined using the commercially available Active Inhibin B kit (Diagnostic Systems Laboratory; Webster, TX). This ELISA was validated for use with rhesus monkey serum in our laboratory. Parallel displacement curves were obtained by comparing serially diluted (1:1 to 1:16) pooled rhesus monkey serum against known INHB concentrations. Recovery of known concentrations of unlabeled INHB added to pooled rhesus monkey serum was 100% with a coefficient of variation of 10%. Inter-assay coefficients of variation for two internal controls were 15% and 9%. The intra-assay coefficient of variation was always less than 10%.

Statistical Analyses:

Descriptive statistics were conducted using SAS Statistical software (Cary, NC). For each ORT we examined the relationship between individual hormone measurements and COS outcome. Number of correct and incorrect predictions (% accuracy) for each ORTs was determined overall for young and old monkeys. Chi-square analyses were performed to evaluate categorical data with ORT predictions compared to actual COS results. Significance was determined at $p < 0.05$.

RESULTS:

Calorie Restriction and the ORTs

Results were similar between control and CR monkeys at each age group and COS classification. Figures 3.1A and B show the mean ± SE for d3 FSH and INHB levels, respectively, for YCON, YCR, OCON (COS+/-) and OCR (COS+/-). As expected, Young monkeys exhibited lower FSH and higher INHB levels as compared to old monkeys. Figures 3.1C and D show the mean ± SE for the CCCT using FSH and INHB levels, respectively, for YCON, YCR, OCON (COS+/-) and OCR (COS+/-). Figures 3.1E, F and G show the mean ± SE for the EFORT using E2, FSH and INHB levels, respectively.

d3 FSH

Figure 3.2 A and B provide the individual d3 FSH values for young and old monkeys, respectively with COS outcome listed. Figure 3.2C shows the ratios of correct to incorrect predictions of CO response. Using the listed criteria for d3 FSH alone, we found an overall 59% accuracy in predicting COS outcome. Day 3 FSH predictions, however, were significantly different ($p < 0.01$) from the actual COS outcomes.

d3 INHB

As anticipated, d3 INHB levels were significantly lower in old as compared to young monkeys, with no diet effects. Figures 3.3A and B reveal the individual d3 INHB values for young and old monkeys, respectively, with COS outcome indicated. Figure 3.3C shows the distribution of correct and incorrect predictions of COS outcome based on the d3 INHB criteria described above. Overall, d3 INHB was 77% accurate in predicting COS outcome. Day 3 INHB predictions, were found to be similar ($p > 0.01$) to actual COS outcomes.

CCCT

A number of hormone markers were evaluated in conjunction with administration of CCCT. Figures 3.4A and B show the individual change in FSH with CCCT. Figure 3.4C shows the ratio of correct to incorrect predictions. Using FSH with the CCCT, we found a 45% accuracy in predicting COS outcome which is lower than using d3 FSH alone (59%). Figures 3.5A and B demonstrate the individual change in INHB with CCCT. Figure 3.5C shows the number of

correct and incorrect COS outcome predictions. CCCT with INHB was 59% accurate which was also lower than d3 INHB alone (77%). Predictions using CCCT with FSH or INHB were both significantly different ($p < 0.01$) from the actual COS outcomes.

Modifying the CCCT to include a second hormone, E2 improved both FSH and INHB prediction accuracy to 77% and 82%, respectively. Figure 3.6A presents the fold change in E2 following CCCT. E2 values were consulted after using d3 FSH or d3 INHB. COS- predictions were then confirmed or refuted, based on fold change in E2. Predictions made using the modified CCCT with both FSH and INHB were found to be similar ($p > 0.01$) to actual COS outcomes.

EFORT

E2, FSH and INHB were evaluated in conjunction with the EFORT. Figures 3.7A and B indicate the individual changes in E2 following the EFORT and the overall accuracy of the hormone in predicting COS outcome (65%). Figures 3.8A and B depict the individual changes in FSH following the EFORT and the overall prediction accuracy for FSH (55%). Figures 3.9A and B show the individual fold change in INHB following EFORT, with a prediction accuracy of 80% (Figure 3.6C). Only predictions made by EFORT using INHB were found to be similar ($p > 0.01$) to actual COS outcomes.

COS

Table 3.6 presents the distribution of COS outcome. All young monkeys had a successful COS (COS+), regardless of diet. Four out of nine (44%) OCON and three out of five (60%) of OCR monkeys had a COS+. Figure 3.10A is a representative profile of a COS+; and Figure 3.10B is a representative profile of a COS-. Accompanying ultrasounds are also presented. .

DISCUSSION:

Elevated FSH is often considered the hallmark effect of ovarian aging and is commonly used in clinical practices to make medical decisions with regard to both pregnancy and/or menopause. With the development of the INHB assay, various reports have suggested that INHB may actually be a more sensitive index for ovarian function and reserve (Klein et al., 1998; Santoro, 2005; Soules et al., 1998). Despite the publication of such reports, INHB levels are still not as widely used as FSH in clinical settings (Klein et al., 2004; Robertson and Burger, 2002; Welt et al., 1999).

Our data consistently find INHB to be superior to FSH in predicting COS outcome, regardless of ORT (Table 3.7). In fact, our results show that the best diagnostic test for predicting COS outcome was the CCCT using INHB values in conjunction with fold change in E2 (82%). These results are in agreement with human studies that have shown INHB to be better than FSH in predicting response to follicle stimulation. Figure 3.11 shows a flow chart depicting the modified CCCT with INHB.

Anti-mullerian hormone (AMH) has recently been suggested as another quality indicator of ovarian reserve (van Rooij et al., 2002; Visser et al., 2006). AMH is produced by the granulosa cells and may be measured in serum (Al-Qahtani et al., 2005; Tremellen et al., 2005). There are limited data evaluating AMH as a potential ORT in women; however, it has not yet been validated in nonhuman primates (Penarrubia et al., 2005; Themmen, 2005).

This is the first report using and comparing ORTs in monkeys. We evaluated the hormonal response to the selected ORTs in both young and old, and control and CR-fed monkeys. There were no differences between groups. Similar findings were reported by our group when analyzing menstrual cycling data (Wu et al., 2004). Although we have yet to determine the effects of CR on delaying menopause, we believe that 30% CR does not inhibit normal ovarian function. Furthermore, based on the results from the ORTs, we would not anticipate any differences with COS between the groups.

Ovarian Reserve Tests

We were surprised to find that both d3 FSH and d3 INHB alone had better predictive value as compared to the CCCT. A couple of obvious factors may be contributing. Clomiphene citrate has a bitter flavor and unlike with human patients, it must be mixed or hidden in a food source when administered to monkeys. With finicky eaters compliance is difficult to assure, even in a laboratory setting. Secondly, the level of clomiphene citrate we used was based on an initial study in which older animals were tested for their response to the

drug (data not shown). Among the young animals, there were several animals where INHB and/or E2 levels dropped precipitously and unexpectedly after administration of the drug. This could be attributed to stress or more likely, an endocrine response to a high level of stimulation.

Lupron (leuprolide acetate) is a GnRH agonist in the short-term, but is often used to inhibit endogenous production of GnRH due to its ability to shut down hypothalamic production of the hormone with continuous application (Badaru et al., 2006). In future studies, we may need to consider titration of clomiphene citrate with different doses at different ages. In a younger animal, levels of clomiphene citrate that are too high may result in the reverse of the desired effect: inhibition rather than stimulation of pituitary and ovarian hormones.

Marut and Hodgen (Marut and Hodgen, 1982) also observed declining levels of E2 in monkeys administered 25 mg clomiphene citrate daily for 5 days, despite elevated levels of FSH. The authors suggested that clomiphene citrate acted in an anti-estrogenic fashion, imparting ovarian refractoriness. Our doses of clomiphene citrate were higher (50 mg daily for 5 days) and we did not observe this same hormone pattern consistently, rather only in select females. This finding further underlines the need to titrate appropriate dosages of clomiphene citrate. Although clomiphene citrate is believed to act by blocking estrogen receptors, the exact mechanism of action is not fully understood. Perhaps in future studies a pure estrogen antagonist would provide more consistent results. With regard to the continued use of the CCCT in rhesus

monkeys, however, use of multiple hormones (day 3 INHB pre-screen and fold change in E2) greatly improves the prediction accuracy of the ORT.

EFORT with INHB showed a predictive accuracy of 80%, very close to the CCCT with INHB. The major advantage to the EFORT is timing. Using the INHB kit (DSL, Webster, TX), hormone assay results are possible within 24 hours. Therefore, the EFORT may be performed with plans to convert to a full COS conditionally, based on the results of the EFORT. This would combine both the ORT and COS into one cycle. Additionally, the EFORT may provide an advantage in situations where oral medication is precluded and injections may be logistically more practical. The primary disadvantage to this method is financial. Should the EFORT predict a COS- the hormone injections would be purely diagnostic. The cost of FSH injections is not trivial and must be weighed against the risk of waiting, especially when dealing with older or genetically valuable animals.

The results for d3 INHB alone cannot be ignored, however, even with the lower prediction accuracy of 77%. As explained previously, clomiphene citrate has an offensive taste and may be difficult to administer in captive or zoo settings. Furthermore, d3 INHB requires only a single blood sample. We were not entirely surprised to find that INHB was more accurate than FSH in predicting COS outcome. This has been shown in human data as well (Danforth et al., 1998; Klein et al., 1996c; Welt et al., 1999). INHB is an ovarian hormone and may be considered a more direct measure of ovarian function than pituitary FSH. Measurement of INHB also has some advantages; INHB enzyme-linked

immunosorbant assay (ELISA) kits are commercially available and relatively easy to perform. Furthermore, there are currently only two manufacturers of the INHB ELISA and they both use the same set of standards. Therefore, INHB values may be easily compared between laboratories.

For rhesus monkeys, the cost for the clomiphene citrate in a CCCT is approximately $3 and the cost for the hormone injections necessary for a COS is approximately $1,000. Although we as scientists strive to perform the best research at all times, it is not always practical to do so. Therefore, it is important to understand the relative efficacy of different ORTs, along with their advantages and disadvantages. It is then possible to make an informed decision with regard to the individual animals, housing situations and technical staff.

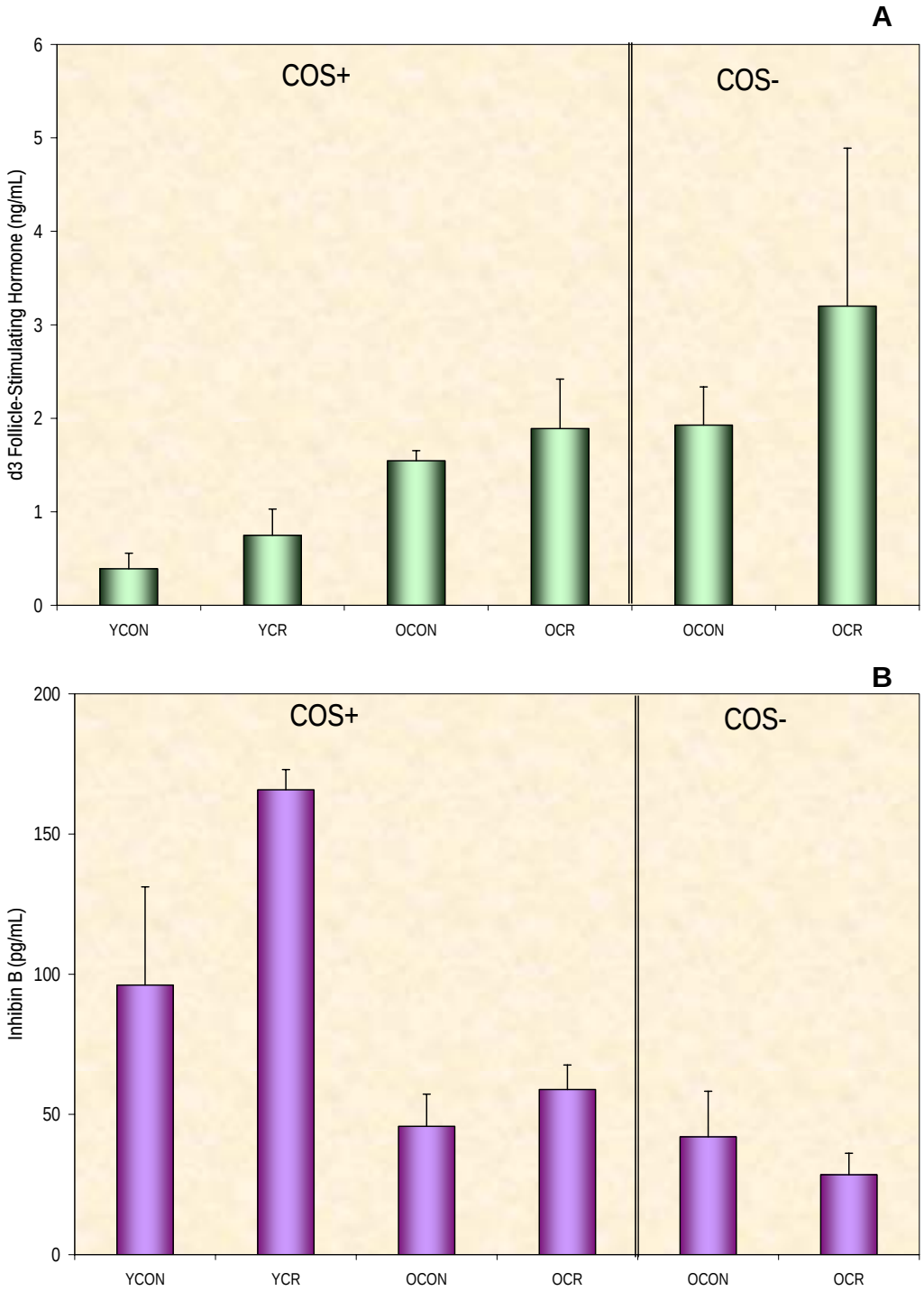
Although ORTs have been in practice in human clinics for many years now, this is the first study addressing their potential use in nonhuman primates. Proper development of ORTS in nonhuman primates can still benefit the human medical community with regard to the identification of reproductively comparable animal models to complement human research.

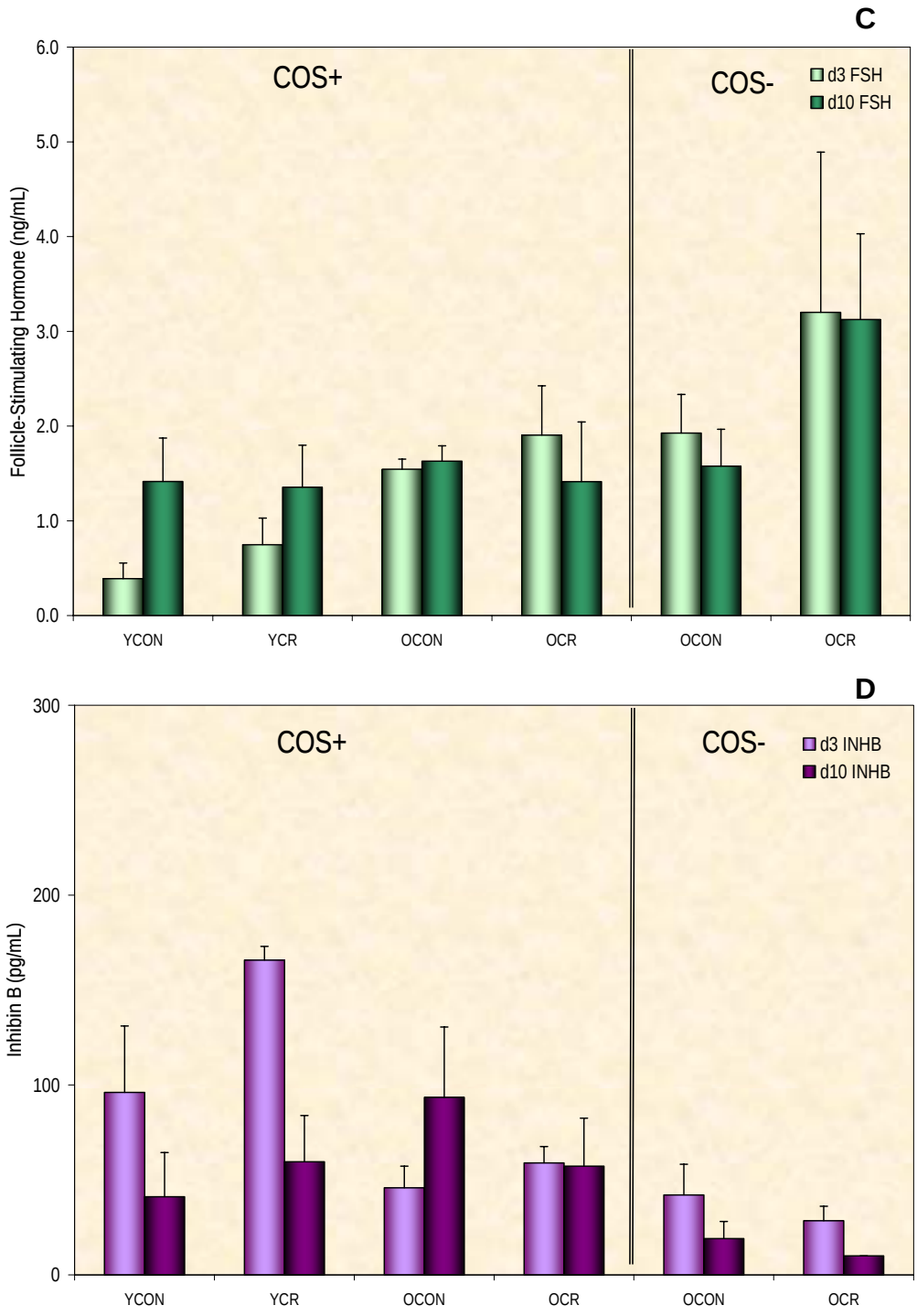
Finally, it important not to discount the value of finding and using the best hormone index with ORTs. Although FSH has long been known to be an early marker of ovarian decline, INHB may be a more sensitive and better predictor of COS outcome. The development of commercially available INHB ELISA kits has made it possible to easily measure INHB without the added precautions necessary when using radioactive isotopes (as with most FSH assays).

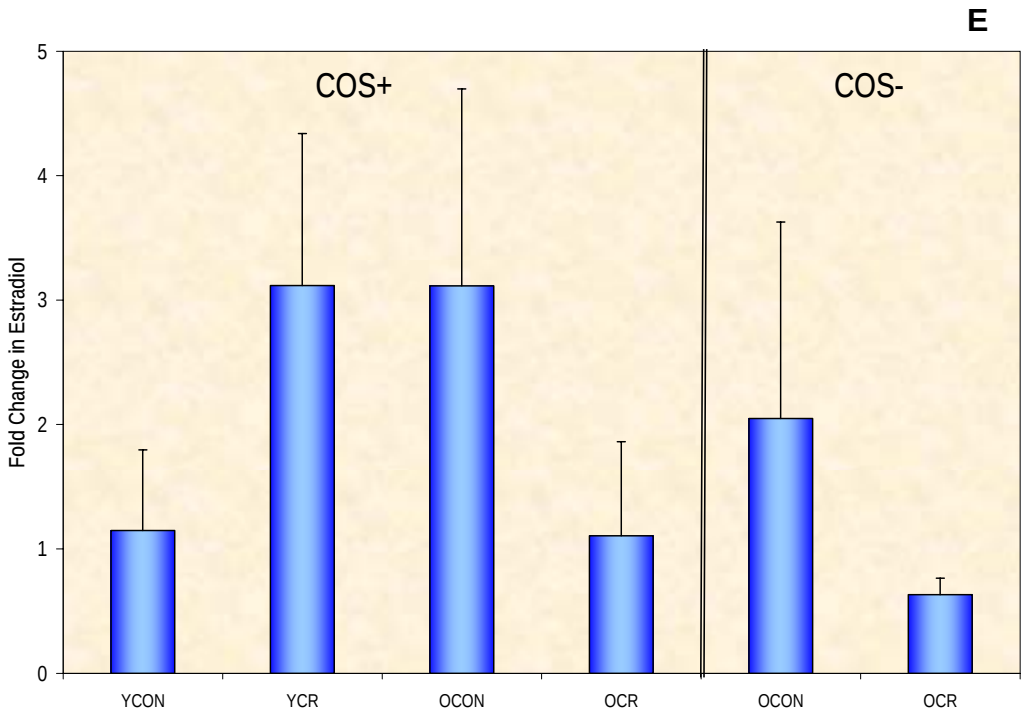
Therefore, use of INHB may be more efficacious than FSH as a diagnostic tool both in humans and monkeys.

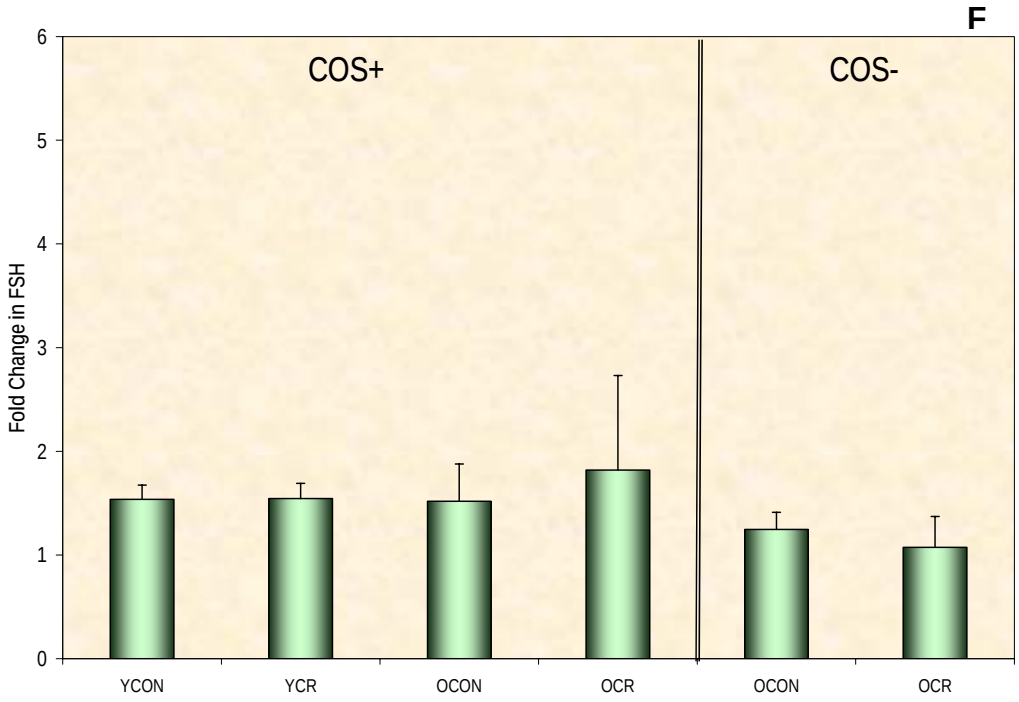
ACKNOWLEDGMENTS

This research was support by NIH AG21382-01, OHSU RR00163 and the Sigma Xi Grants in Aid of Research. We would like to thank the members of the Oregon National Primate Research Center (ONPRC): Diana Takahashi, Maralee Lawson, Dr. David Hess, Dr. Frank Koegler, Dr. Francis Pau, Dr. John Fanton, Dr. Ted Hobbs, Megan Martin, Lindsay Pranger and Olivia Thoene.

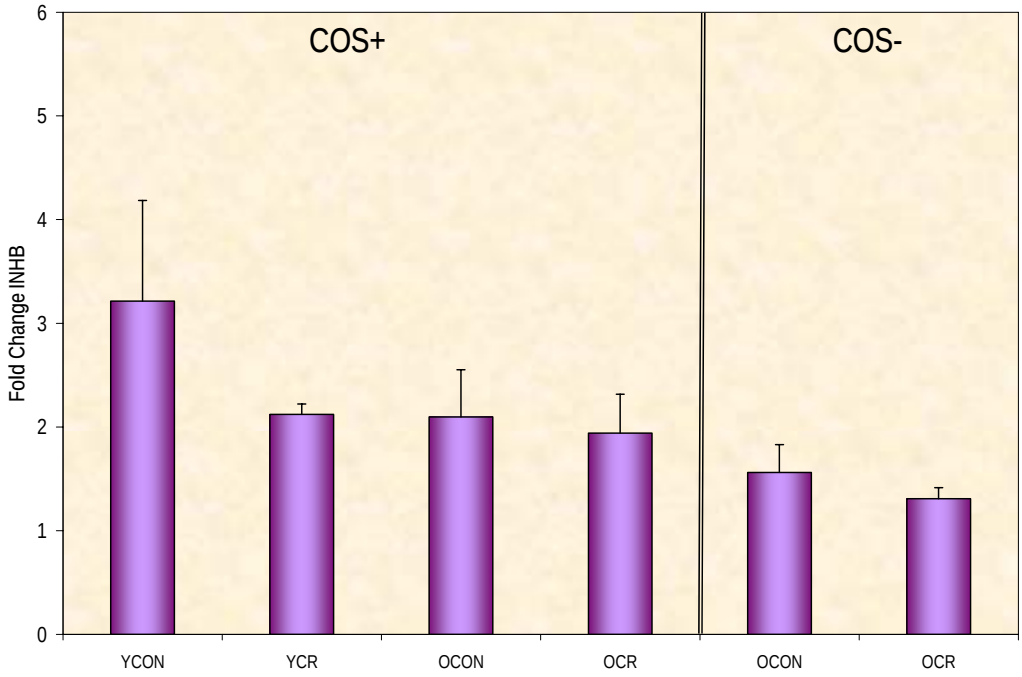

A
COS+
COS-
d3 Follicle-Stimulating Hormone (ng/mL)
0
1
2
3
4
5
6
YCON
YCR
OCON
OCR
OCON
OCR
B
COS+
COS-
Inhibin B (pg/mL)
0
50
100
150
200
YCON
YCR
OCON
OCR
OCON
OCR


C
COS+
COS-
d3 FSH
d10 FSH
Follicle-Stimulating Hormone (ng/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
YCON
YCR
OCON
OCR
OCON
OCR
D
COS+
COS-
d3 INHB
d10 INHB
Inhibin B (pg/mL)
300
200
100
0
YCON
YCR
OCON
OCR
OCON
OCR


E
COS+
COS-
Fold Change in Estradiol
0
1
2
3
4
5
YCON
YCR
OCON
OCR
OCON
OCR


F
COS+
COS-
Fold Change in FSH
0
1
2
3
4
5
6
YCON
YCR
OCON
OCR
OCON
OCR

G


COS+
COS-
Fold Change INHB
0
1
2
3
4
5
6
YCON
YCR
OCON
OCR
OCON
OCR

H

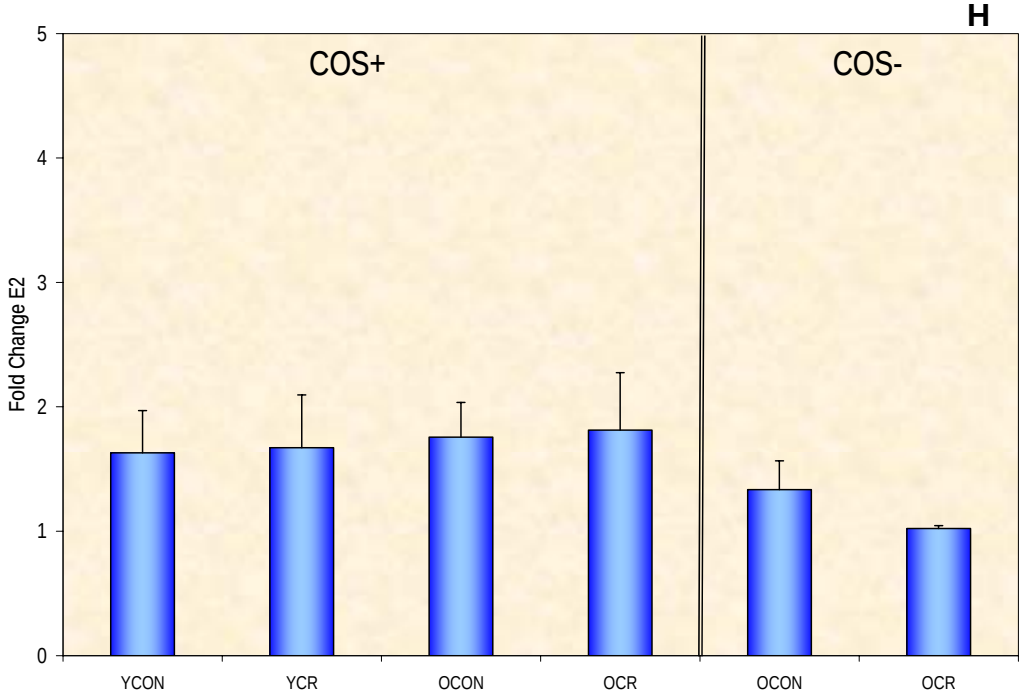
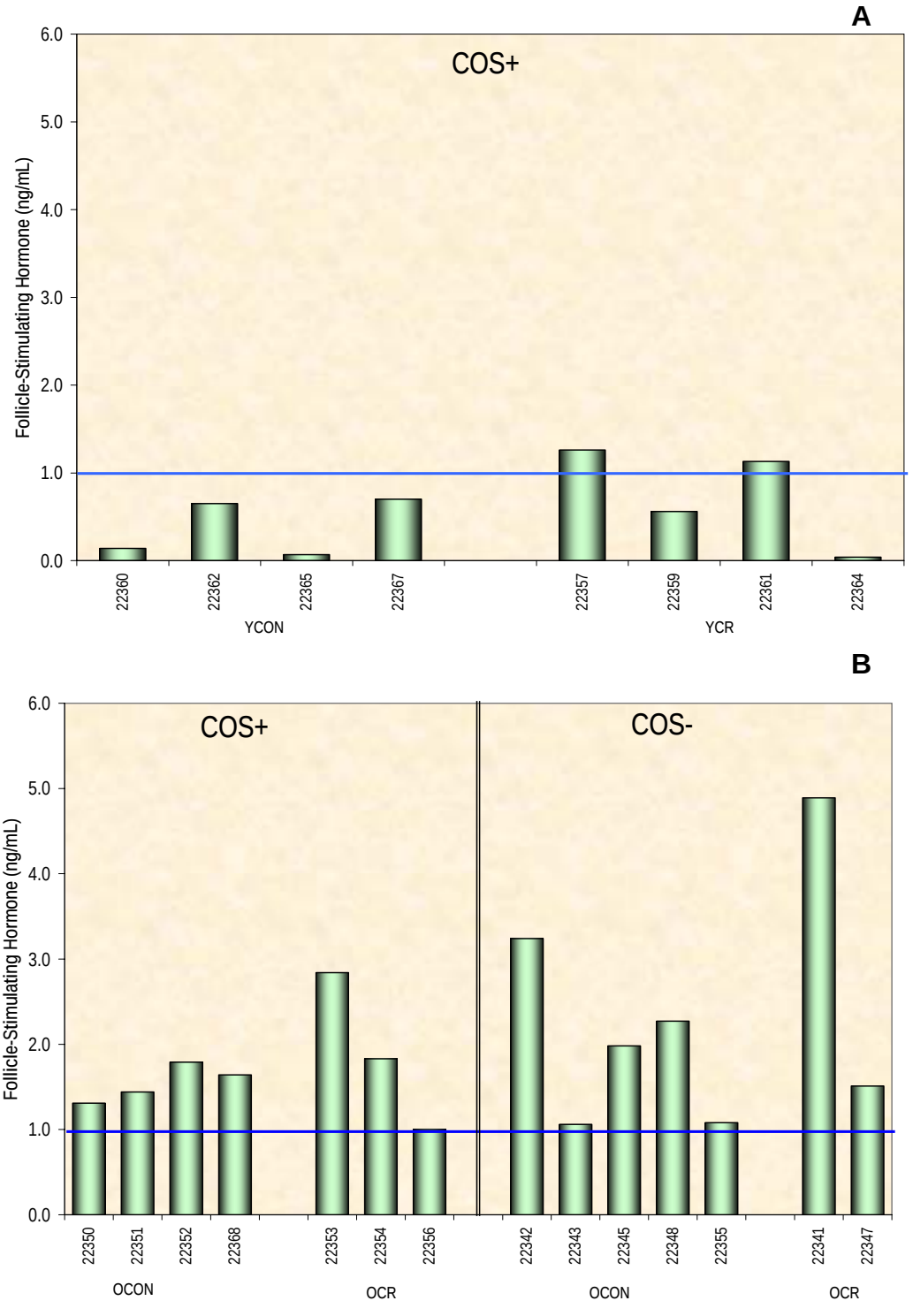

COS+
COS-
Fold Change E2
0
1
2
3
4
5
YCON
YCR
OCON
OCR
OCON
OCR

Figure 3.1 Mean hormone levels with ORTs. Results of ovarian reserve tests (ORTs) for Young Control (YCON), Young CR (YCR), Old Control (OCON) and Old CR (OCR) with COS outcome indicated: successful COS (COS+) or unsuccessful COS (COS-). A) d3 Follicle-Stimulating Hormone (FSH). B) d3 Inhibin B (INHB). C) d3 and d10 FSH during Clomiphene Citrate Challenge Test (CCCT). D) d3 and d10 INHB during CCCT. E) Fold change in Estradiol (E2) during CCCT. F) Fold change in FSH during EFORT. G) Fold change in INHB during EFORT. H) Fold change in E2 during EFORT. No diet effects were observed.


A
COS+
Follicle-Stimulating Hormone (ng/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
22360
22362
22365
22367
22357
22359
22361
22364
YCON
YCR
B
COS+
COS-
Follicle-Stimulating Hormone (ng/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
22350
22351
22352
22368
22353
22354
22356
22342
22343
22345
22348
22355
22341
22347
OCON
OCR
OCON
OCR

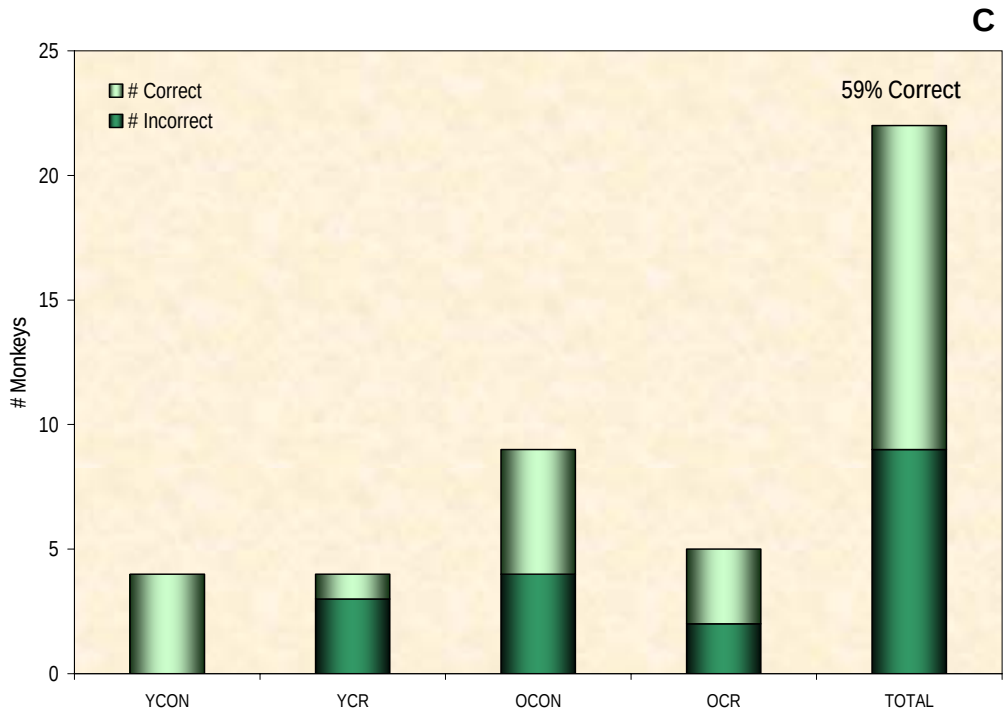
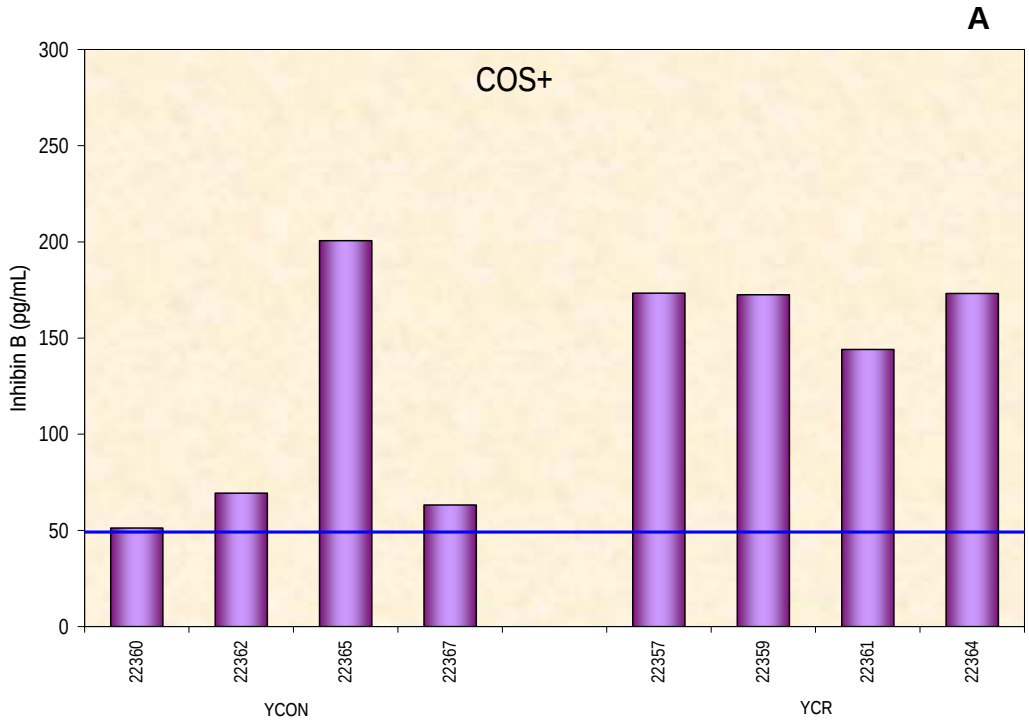
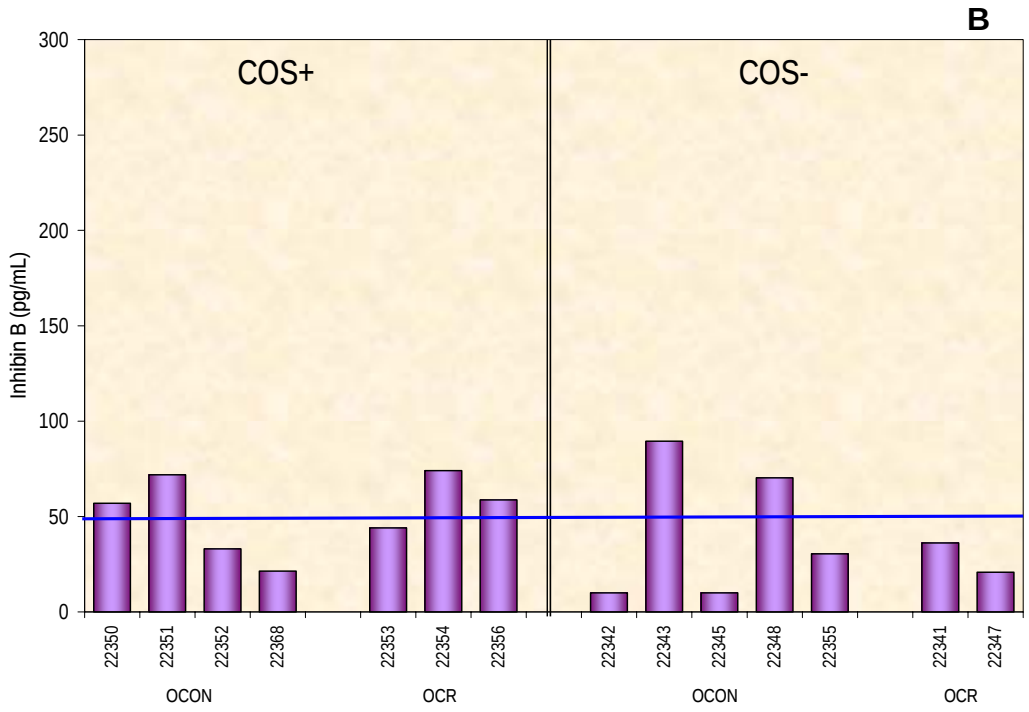


Figure 3.2 Day 3 Follicle-Stimulating Hormone (FSH). A) Individual d3 FSH for young monkeys. B) Individual d3 FSH for old monkeys, with retrospective COS+ or COS- indicated. C) Number correct and incorrect COS outcome predictions with overall percent accuracy indicated. Day 3 FSH predictions did not predict ($p > 0.05$) actual COS outcomes.


A
COS+
Inhibin B (pg/mL)
300
250
200
150
100
50
0
22360
22362
22365
22367
22357
22359
22361
22364
YCON
YCR


B
COS+
COS-
Inhibin B (pg/mL)
300
250
200
150
100
50
0
22350
22351
22352
22368
22353
22354
22356
22342
22343
22345
22348
22355
22341
22347
OCON
OCR
OCON
OCR

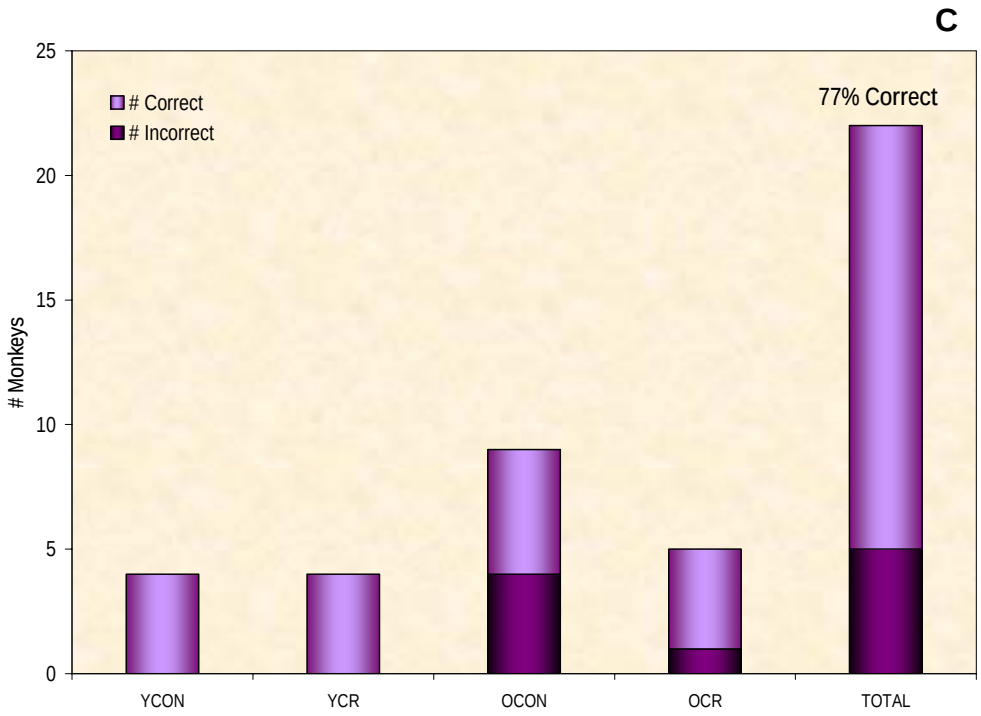
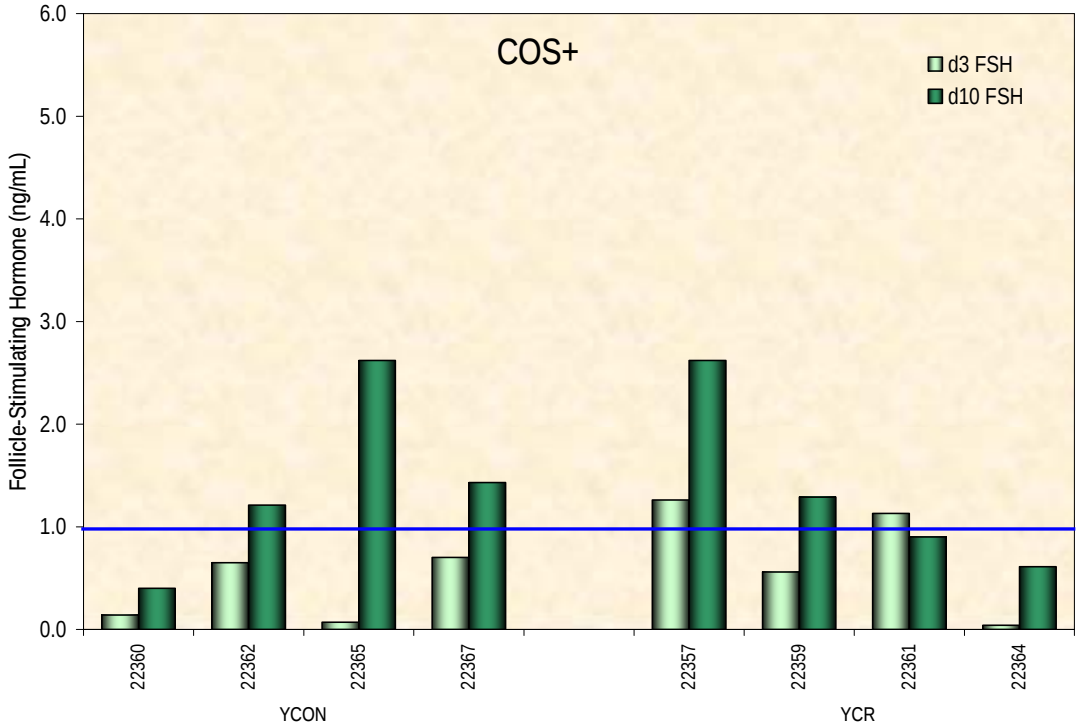
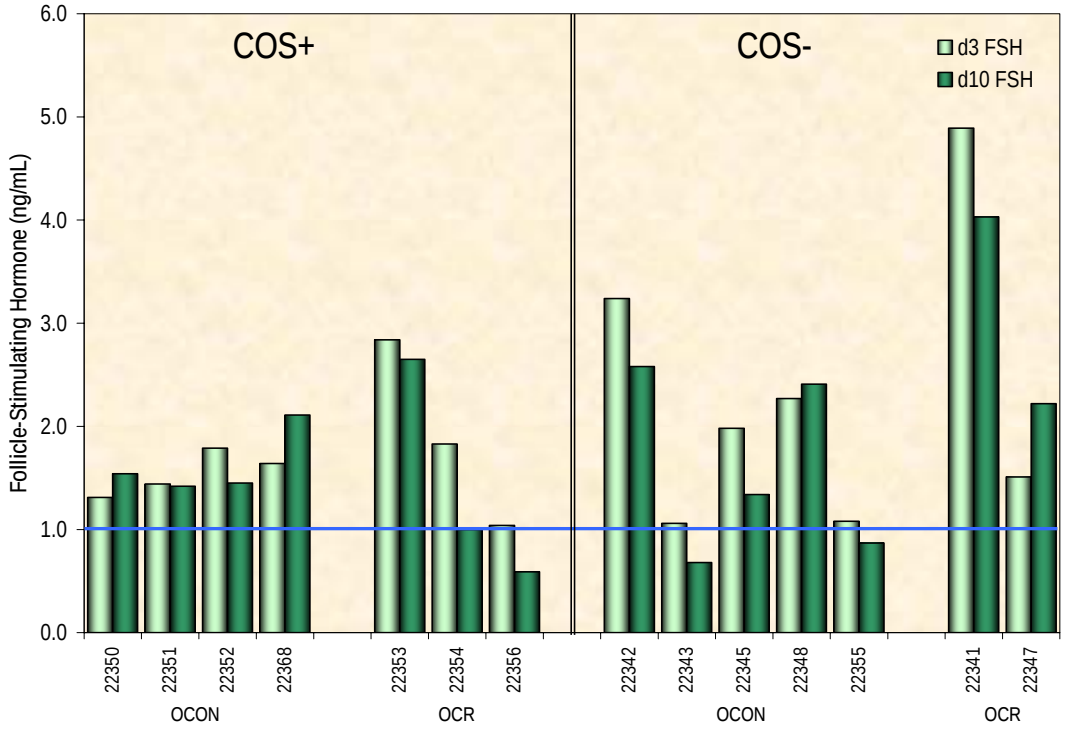


Figure 3.3 Day 3 Inhibin B (INHB). A) Individual d3 INHB for young monkeys. B) Individual d3 INHB for old monkeys, with retrospective COS+ or COS- indicated. C) Number correct and incorrect COS outcome predictions with overall percent accuracy indicated. Day 3 INHB predictions were marginally similar ($p = 0.06$) to actual COS outcomes.

A


COS+
d3 FSH
d10 FSH
Follicle-Stimulating Hormone (ng/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
22360
22362
22365
22367
22357
22359
22361
22364
YCON
YCR

B


COS+
COS-
d3 FSH
d10 FSH
Follicle-Stimulating Hormone (ng/mL)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
22350
22351
22352
22368
22353
22354
22356
22342
22343
22345
22348
22355
22341
22347
OCON
OCR
OCON
OCR

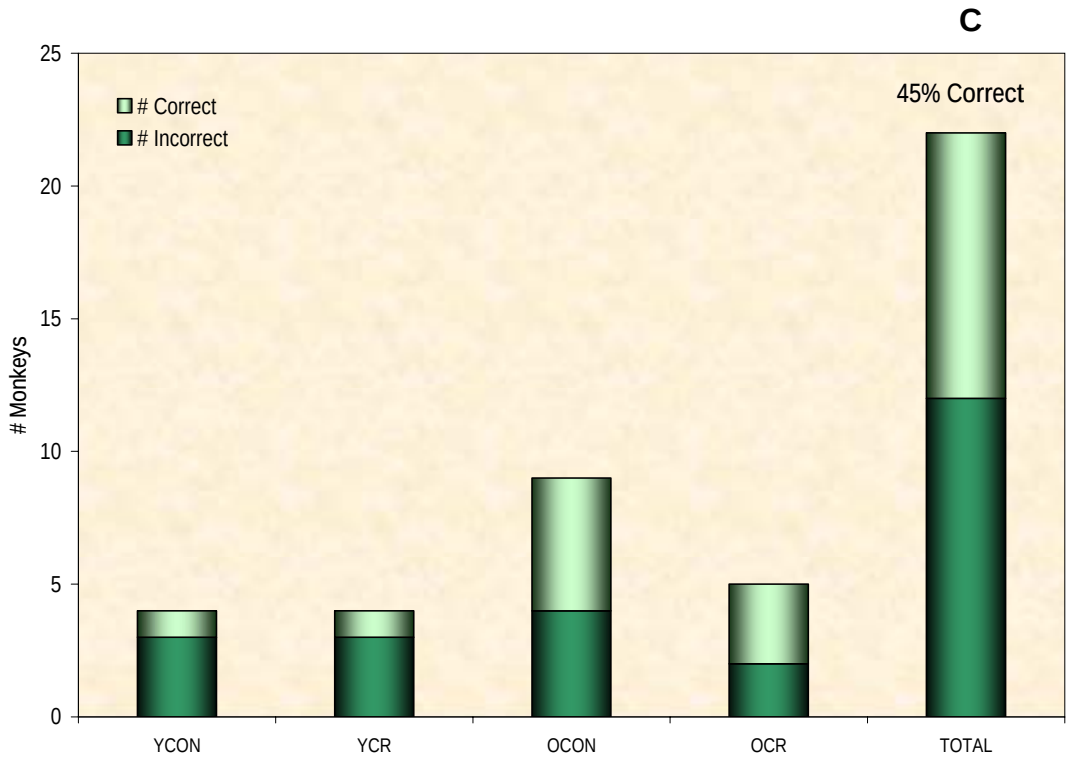
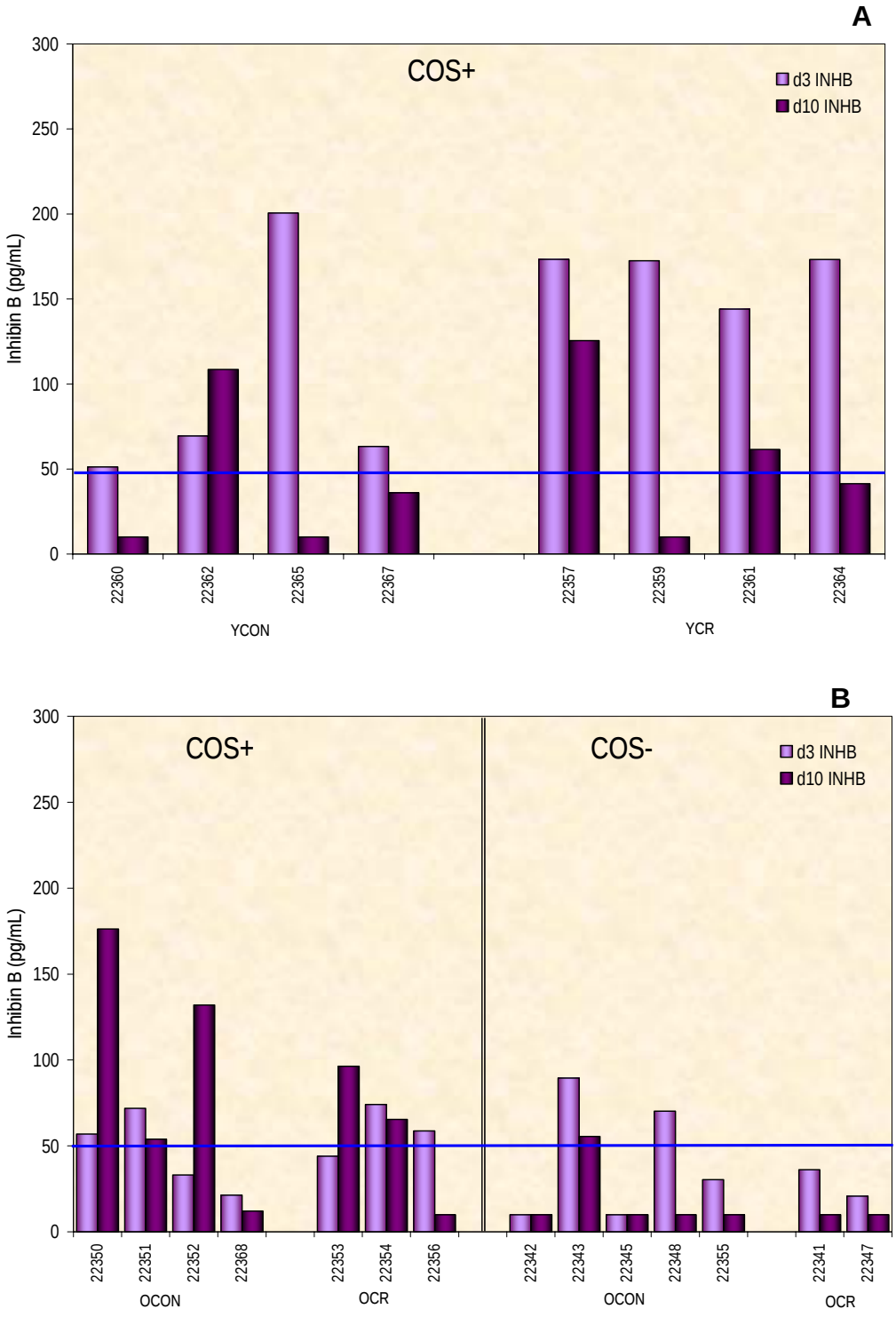


Figure 3.4 Clomiphene Citrate Challenge Test (CCCT) using Follicle-Stimulating Hormone (FSH). A) Individual FSH with CCCT for young monkeys on d3 and d10. B) Individual FSH with CCCT for old monkeys on d3 and d10, with retrospective COS+ or COS- indicated. C) Number correct and incorrect COS outcome predictions with overall percent accuracy indicated. CCCT with FSH predictions nearly predicted ($p > 0.05$) actual COS outcomes.


A
COS+
d3 INHB
d10 INHB
Inhibin B (pg/mL)
300
250
200
150
100
50
0
22360
22362
22365
22367
22357
22359
22361
22364
YCON
YCR
B
COS+
COS-
d3 INHB
d10 INHB
Inhibin B (pg/mL)
300
250
200
150
100
50
0
22350
22351
22352
22368
22353
22354
22356
22342
22343
22345
22348
22355
22341
22347
OCON
OCR
OCON
OCR

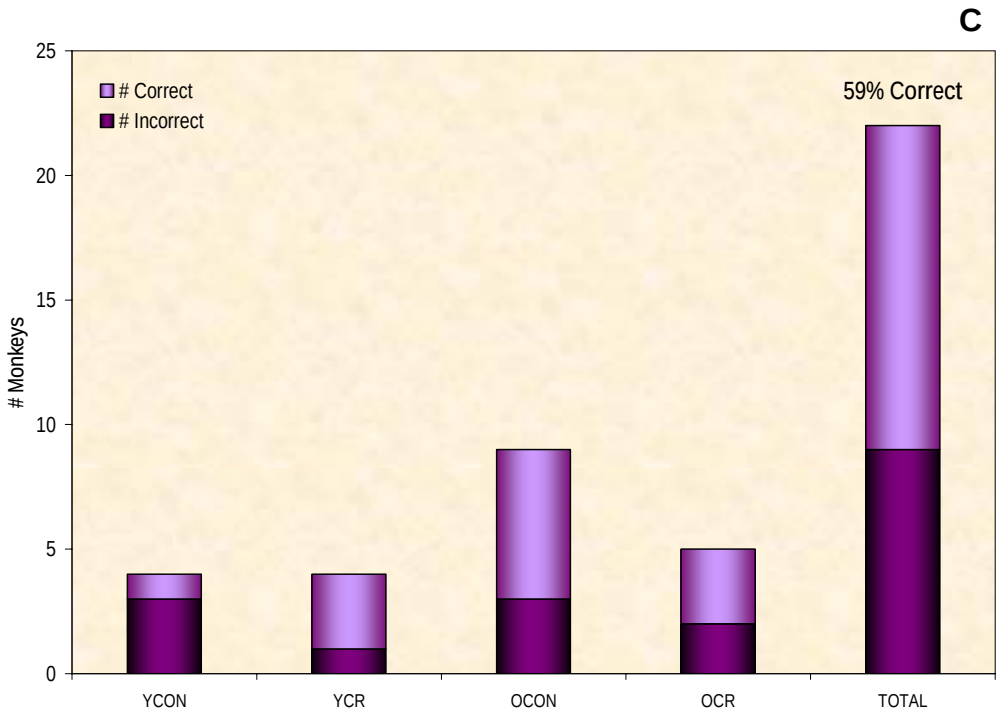
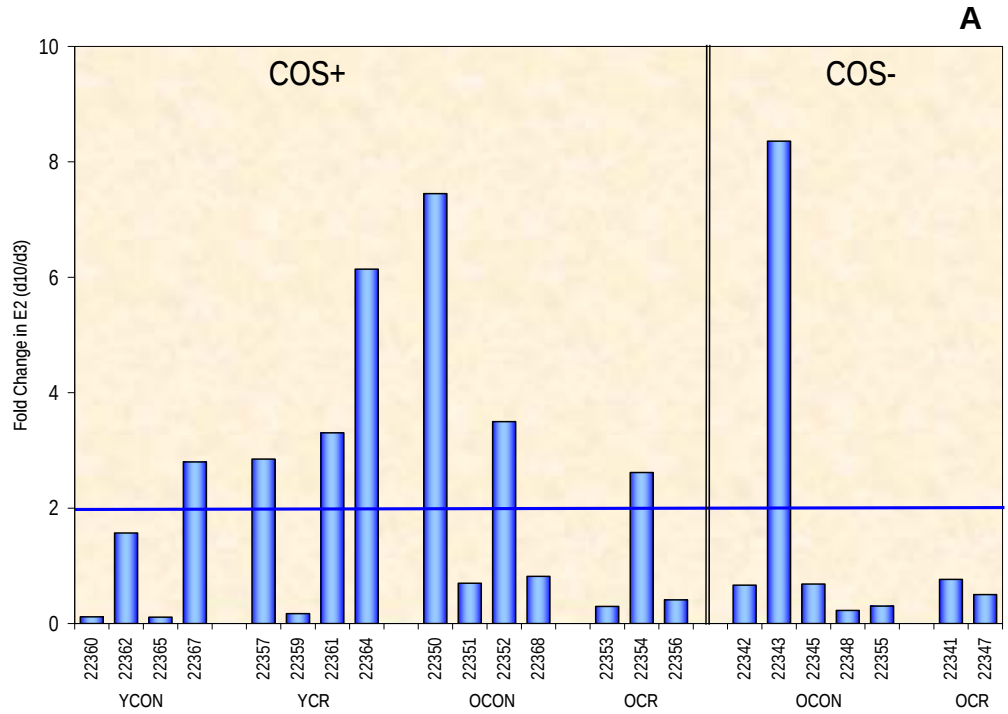
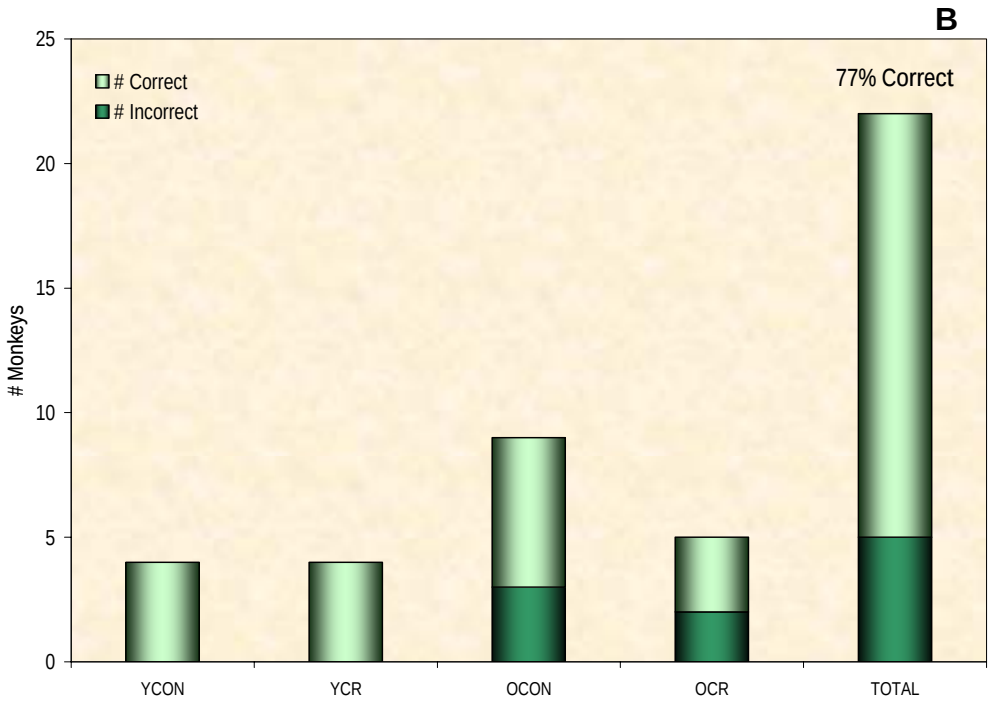


Figure 3.5 Clomiphene Citrate Challenge Test (CCCT) using Inhibin B (INHB). A) Individual CCCT with INHB for young monkeys on d3 and d10. B) Individual CCCT with INHB for old monkeys on d3 and d10, with retrospective COS+ or COS- indicated. C) Number correct and incorrect COS outcome predictions with overall percent accuracy indicated. CCCT with INHB predictions did not predict ($p > 0.01$) actual COS outcomes.


A
COS+
COS-
Fold Change in E2 (d10/d3)
10
8
6
4
2
0
22360
22362
22365
22367
22357
22359
22361
22364
22350
22351
22352
22368
22353
22354
22356
22342
22343
22345
22348
22355
22341
22347
YCON
YCR
OCON
OCR
OCON
OCR


B
Correct
Incorrect
77% Correct
Monkeys
25
20
15
10
5
0
YCON
YCR
OCON
OCR
TOTAL

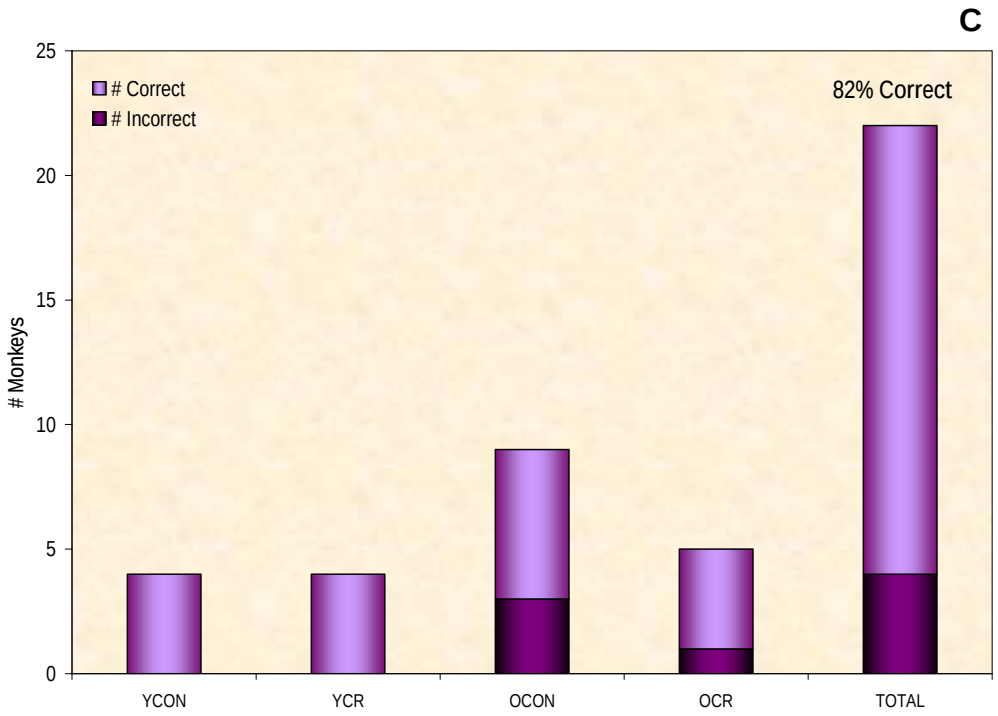
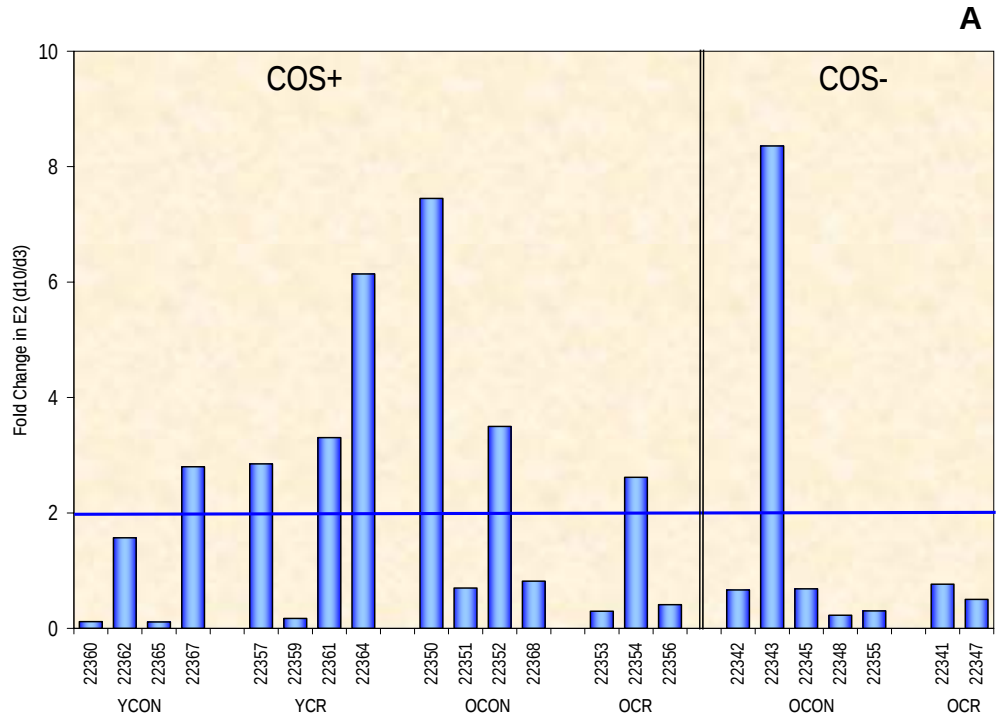


Figure 3.6 Modified Clomiphene Citrate Challenge Test (CCCT) Using Follicle-Stimulating Hormone (FSH) and fold change in Estradiol (E2) or Inhibin B (INHB) and fold change in Estradiol (E2). A) Individual fold change in E2 from d3 to d10, following CCCT. B) Number of correct and incorrect predictions using FSH with CCCT in conjunction with fold change in E2, including overall percent accuracy. The modified CCCT with FSH predicted ($p < 0.05$) actual COS outcomes. C) Number of correct and incorrect predictions using INHB with CCCT in conjunction with fold change in E2, including overall percent accuracy. The modified CCCT with INHB predicted ($p < 0.05$) actual COS outcomes.


A
COS+
COS-
Fold Change in E2 (d10/d3)
10
8
6
4
2
0
22360
22362
22365
22367
22357
22359
22361
22364
22350
22351
22352
22368
22353
22354
22356
22342
22343
22345
22348
22355
22341
22347
YCON
YCR
OCON
OCR
OCON
OCR

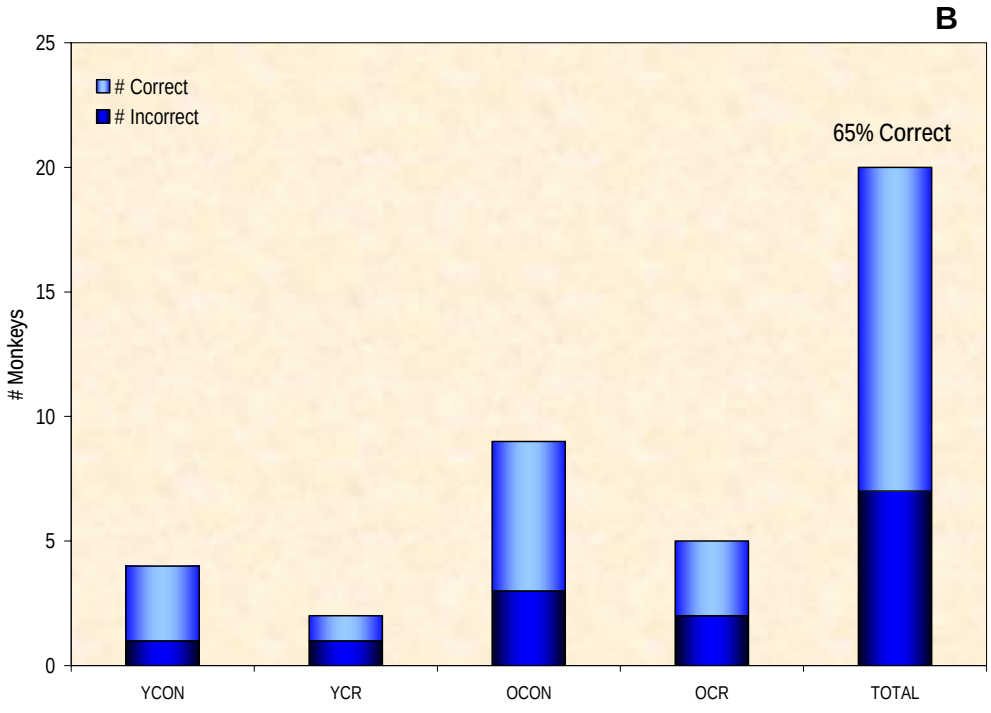
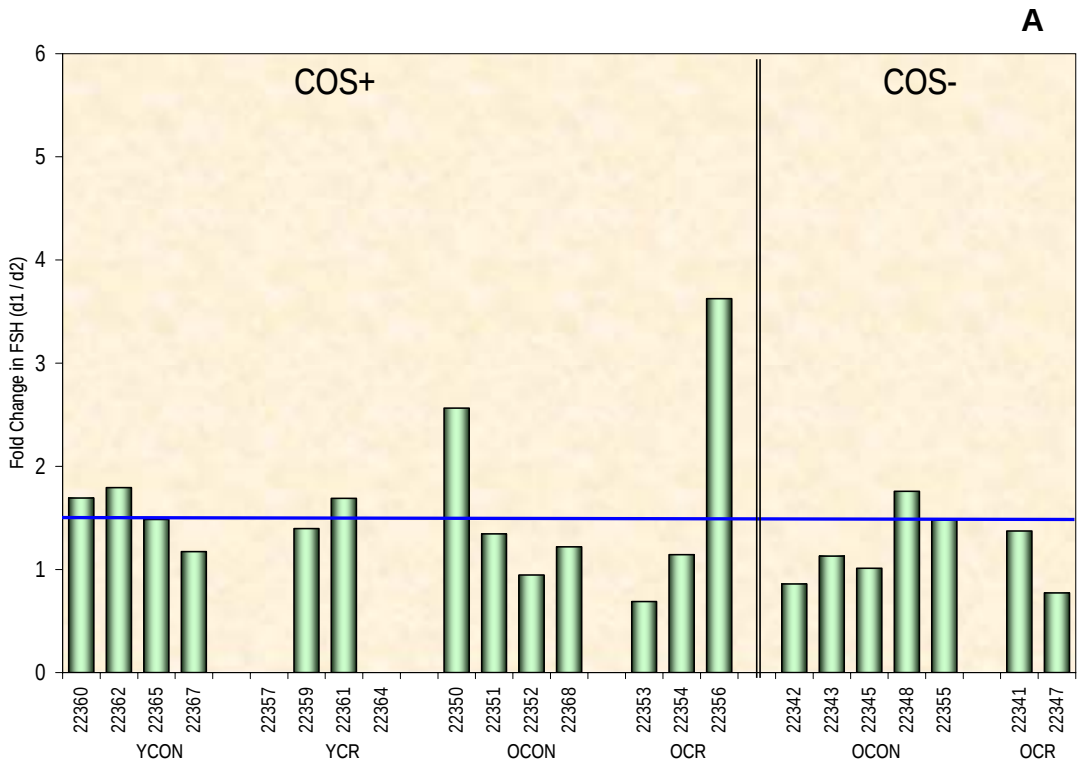

B
Correct
Incorrect
65% Correct
Monkeys
25
20
15
10
5
0
YCON
YCR
OCON
OCR
TOTAL

Figure 3.7 Exogenous Follicle-Stimulating Hormone Ovarian Reserve Test (EFORT) using fold change in Estradiol (E2). A) Individual fold change (increase) in E2 on d1 and d2 following EFORT. B) Number of correct and incorrect predictions using fold change in E2 with EFORT, including overall percent accuracy. EFORT with E2 did not predict ($p > 0.05$) actual COS outcomes.


A
COS+
COS-
Fold Change in FSH (d1 / d2)
0
1
2
3
4
5
6
22360
22362
22365
22367
22357
22359
22361
22364
22350
22351
22352
22368
22353
22354
22356
22342
22343
22345
22348
22355
22341
22347
YCON
YCR
OCON
OCR
OCON
OCR

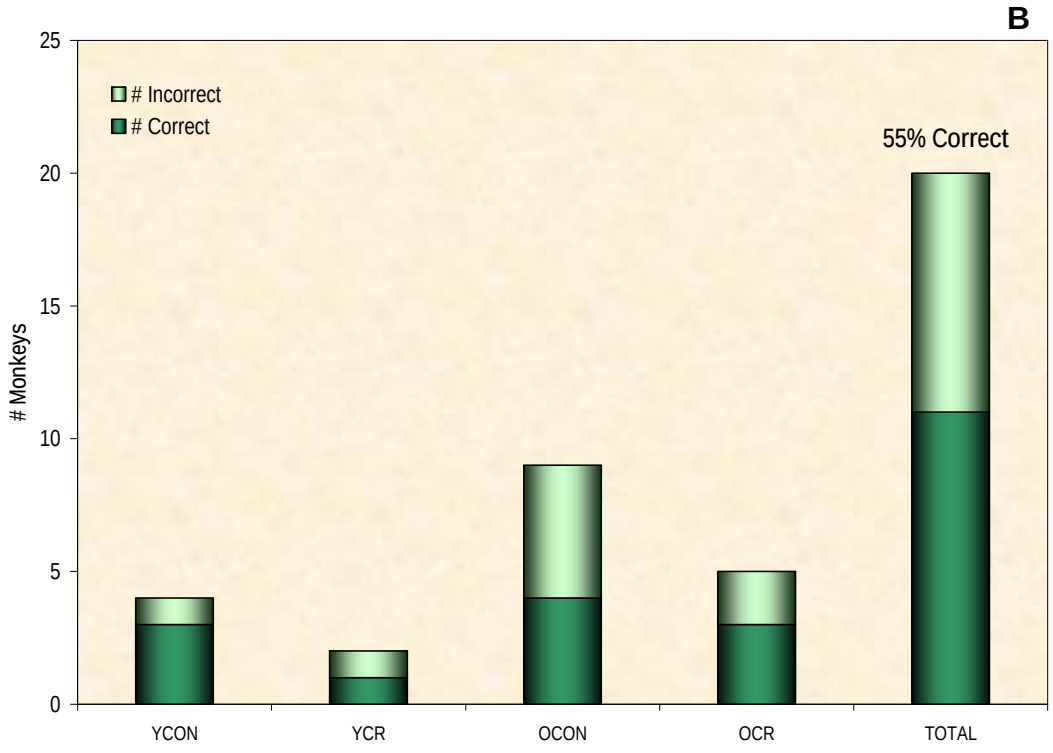
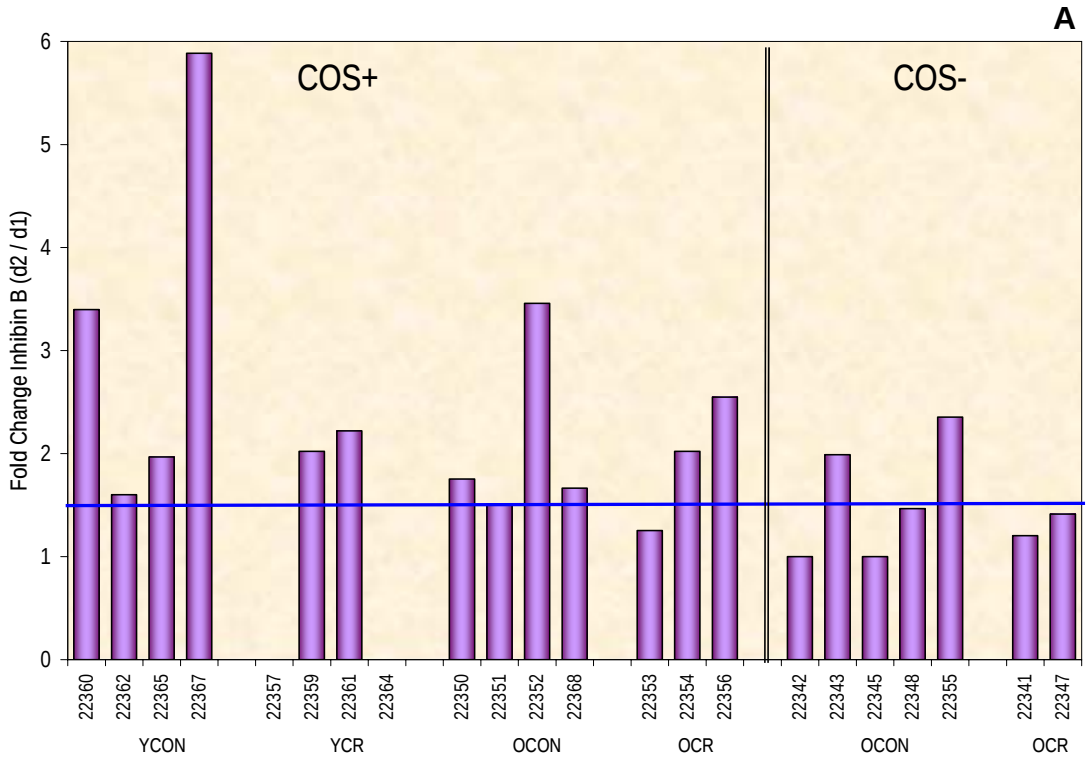

B
Incorrect
Correct
55% Correct
Monkeys
0
5
10
15
20
25
YCON
YCR
OCON
OCR
TOTAL

Figure 3.8 Exogenous Follicle-Stimulating Hormone Ovarian Reserve Test (EFORT) using fold change in Follicle-Stimulating Hormone (FSH). A) Individual fold change in FSH on d1 and d2 following EFORT. B) Number of correct and incorrect predictions using fold change (decrease) in FSH with EFORT, including overall percent accuracy. EFORT with FSH did not predict ($p > 0.05$) actual COS outcomes.


A
COS+
COS-
Fold Change Inhibin B (d2 / d1)
0
1
2
3
4
5
6
22360
22362
22365
22367
22357
22359
22361
22364
22350
22351
22352
22368
22353
22354
22356
22342
22343
22345
22348
22355
22341
22347
YCON
YCR
OCON
OCR
OCON
OCR

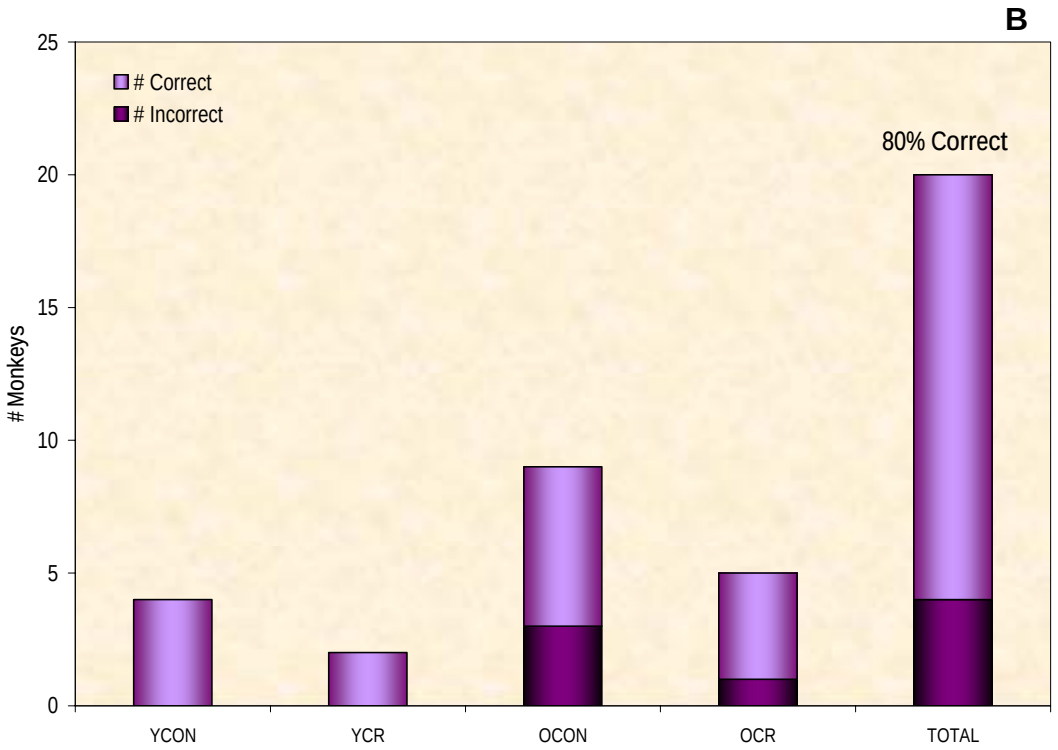
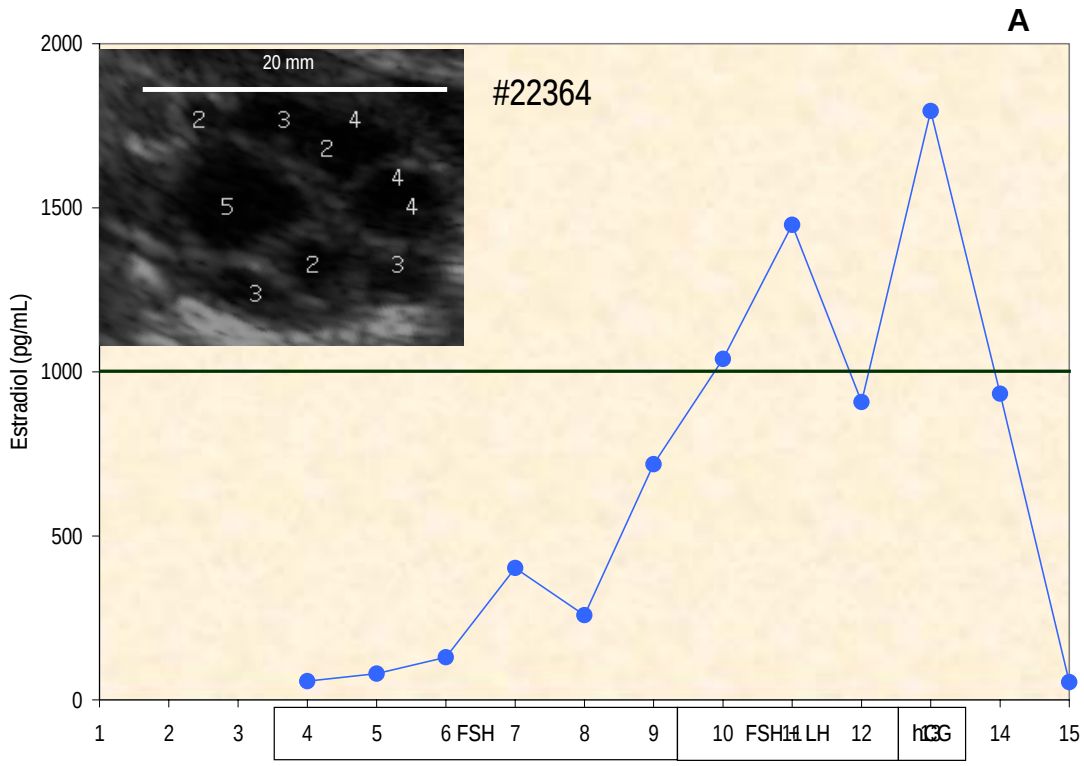

B
Correct
Incorrect
80% Correct
Monkeys
0
5
10
15
20
25
YCON
YCR
OCON
OCR
TOTAL

Figure 3.9 Exogenous Follicle-Stimulating Hormone Ovarian Reserve Test (EFORT) using fold change in Inhibin B (INHB). A) Individual fold change in INHB on d1 and d2 following EFORT. B) Number of correct and incorrect predictions using fold change in INHB with EFORT, including overall percent accuracy. EFORT with INHB predictions nearly predicted ($p = 0.06$) actual COS outcomes.

Table 3.6 Outcome of controlled ovarian stimulation (COS) for young and old monkeys. Young CON (YCON), young CR (YCR), old CON (OCON), old CR (OCR) are indicated with COS outcome: successful (COS+) or unsuccessful (COS-).

ID	Cohort	COS
22360	YCON	+
22362	YCON	+
22365	YCON	+
22367	YCON	+
22357	YCR	+
22359	YCR	+
22361	YCR	+
22364	YCR	+
22350	OCON	+
22351	OCON	+
22352	OCON	+
22368	OCON	+
22353	OCR	+
22354	OCR	+
22356	OCR	+
22342	OCON	-
22343	OCON	-
22345	OCON	-
22348	OCON	-
22355	OCON	-
22341	OCR	-
22347	OCR	-


A
20 mm
#22364
Estradiol (pg/mL)
2000
1500
1000
500
0
1 2 3 4 5 6 FSH 7 8 9 10 FSH 11 LH 12 hCG 14 15

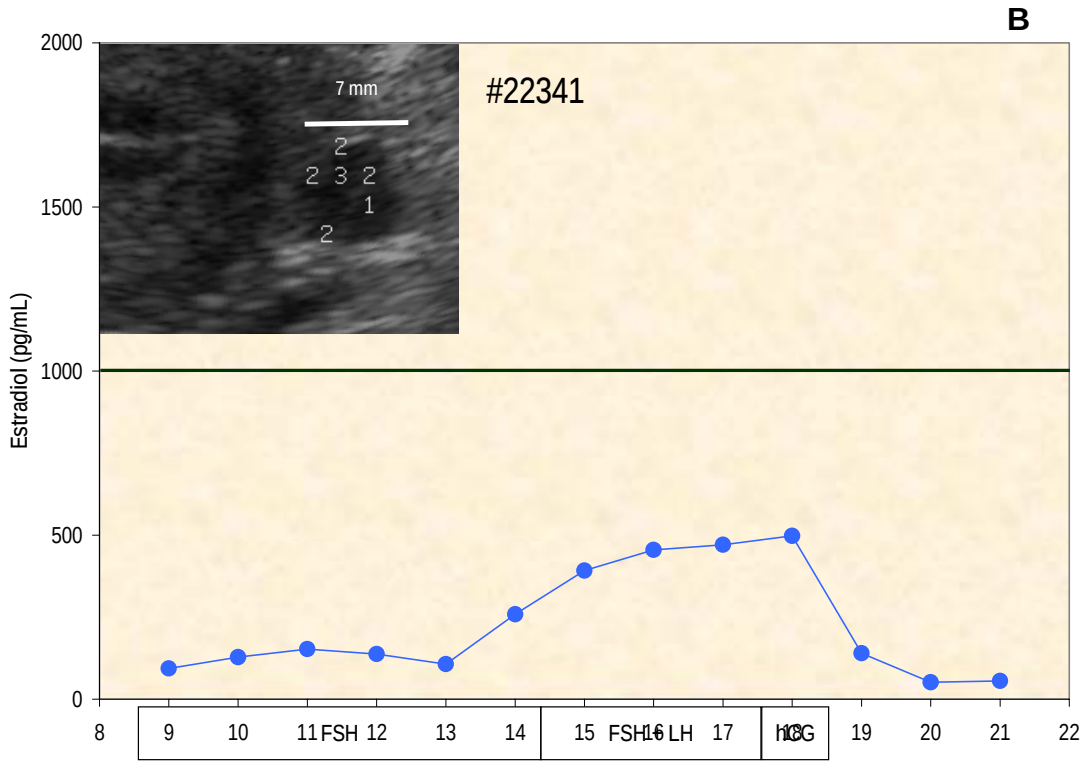

B
7 mm
#22341
Estradiol (pg/mL)
2000
1500
1000
500
0
8 9 10 11 FSH 12 13 14 15 FSH 16 LH 17 hCG 19 20 21 22

Figure 3.10 Representative hormone profiles of a controlled ovarian stimulation (COS). A) Representative estradiol (E2) profile of a successful COS (COS+) with accompanying ultrasound. B) Representative E2 profile of an unsuccessful COS (COS-) with accompanying ultrasound.

Table 3.7 Summary Table of percent accuracy for selected ovarian reserve tests (ORTs). d3 follicle-stimulating hormone (FSH), d3 inhibin B (INHB), clomiphene citrate challenge test (CCCT) with and without the estradiol (E2) modification and the exogenous FSH ovarian reserve test (EFORT). * indicates ORTs that predicted ($p < 0.05$) actual COS outcomes. ** indicates ORTs that nearly predicted ($p = 0.06$) actual COS outcomes.

	% Accuracy in Predicting COS Outcome		
	FSH	INHB	E2
d3	59%	77%**	
CCCT	45%	59%	
CCCT + E2	77%*	82%*	
EFORT	55%	80%**	65%

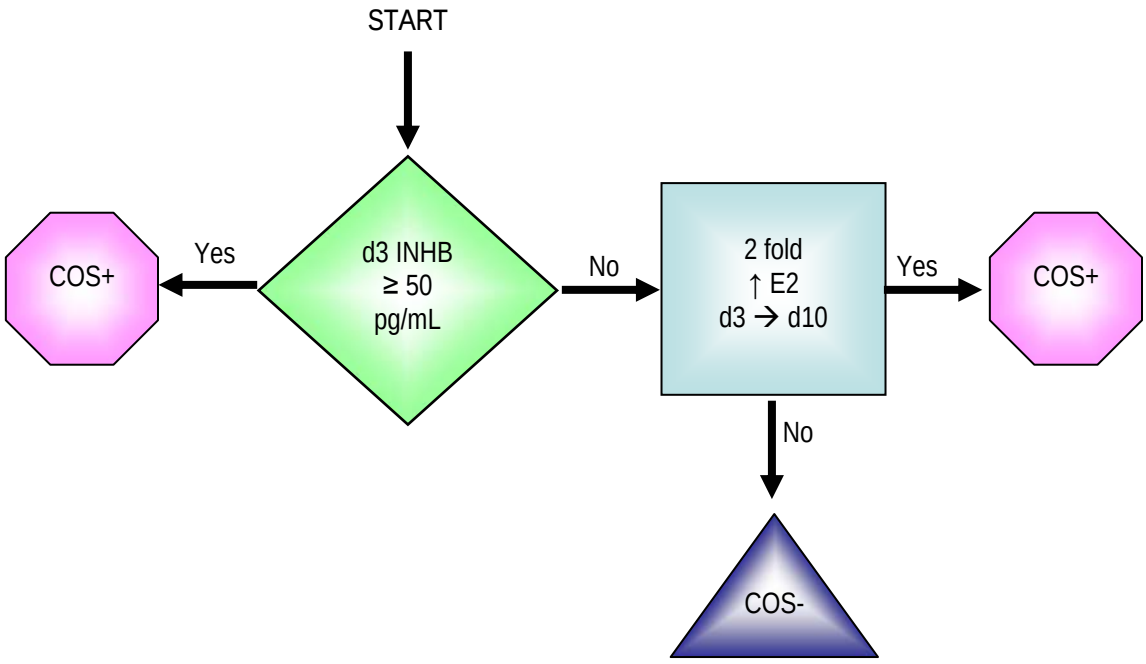


Figure 3.11 Flow chart for Modified CCCT Using Inhibin B. Clomiphene citrate challenge test (CCCT) using inhibin B (INHB) values and fold change in estradiol (82% accuracy). Day 3 INHB serves as an initial screen; values ≥ 50 pg/mL may be excluded from further testing and predict for a successful COS (COS+). If INHB < 50 pg/mL a secondary screen for fold change in E2 determines whether COS outcome will be successful (COS+) or unsuccessful (COS-).

CHAPTER 4

CALORIE RESTRICTION ENHANCES EMBRYOGENESIS IN THE FEMALE RHESUS MONKEY

INTRODUCTION:

The median age of menopause in women is approximately 50 years (Treloar, 1981); however, changes in normal hormone profiles are measurable in women years before clinical menopause (> 1 year without menses). Incidence of aneuploidy, chromosomal abnormalities and miscarriage also increase with age. Furthermore, data collected form human infertility clinics suggest that aging occurs first in the oocytes and secondarily in the uterus (Navot et al., 1991; Navot et al., 1994). Donor eggs collected from young woman may be implanted in uteri of older women and delivered successfully, while the reverse is not necessarily true.

Calorie restriction (CR) has been proven to increase longevity in a number of species, including *C. elegans*, *D. melanogaster*, rats and mice (Smith et al., 2004). The mechanism of action of CR is complex and appears to involve reduced damage incurred by free radical production via oxidative stress (Merry, 2004). This action of CR may provide benefits for a variety of cell types, including possible effect on gametes.

Data on the effects of CR on reproduction are mixed and titration of its effects is necessary. Data from rats and mice are not consistent their findings of CR effect on reproduction. In mice, CR disrupted normal cycling, however

resumption of normal reproductive function occurred with refeeding (Nelson et al., 1985; Nelson et al., 1995). Furthermore, onset of ovarian decline was subsequently delayed as well. Conversely in rats, CR increased reproductive longevity without disrupting normal cycling (Holehan and Merry, 1985a; Merry and Holehan, 1985).

The rhesus monkey *(Macaca mulatta)* provides an ideal model for human reproductive studies in particular with regard to menopause. Rhesus monkeys exhibit menstrual cycles similar to women, approximately 28 days in length (Heape, 1900). The hormone profiles for estradiol (E2), progesterone (P4), follicle-stimulation hormone (FSH), and luteinizing hormone (LH) are also comparable to human cycles. Furthermore, like humans, rhesus monkeys experience the periodic sloughing of the endometrial lining, resulting in menstruation. This is unique to primates and thus essential in studying a true menopause. Despite great interest in the applicability of CR or CR mimetics in humans, there has been little research evaluating its effect on the reproductive system in nonhuman primates. Black and colleagues (Black et al., 2001) did not observe adverse effects with long-term CR in rhesus monkeys (ages 7-27 years) with regard to reproductive hormones measured on menstrual day 5.

Research on the effect of short-term CR in the primate model is ongoing and some of the specific effects of CR on reproductive function are the focus of this paper. This study is the first to evaluate the effects of CR on response to controlled ovarian stimulation (COS), oocyte maturation, fertilization and embryo development in old, peri-menopausal rhesus monkeys.

MATERIALS AND METHODS

Animals and Diet

Rhesus monkeys *(Macaca mulatta)* were housed indoors, with fresh water *ad libitum* under 12L:12D lighting schedule. Monkeys were housed at the Oregon National Primate Research Center (ONPRC), Oregon Health and Sciences University (OHSU; Beaverton, OR). Old, peri-menopausal rhesus monkeys were identified for this study.

Prior to implementation of the CR feeding regimen, monkeys were monitored to determine daily food intake based on *ad libitum* feed availability. The average daily value was then divided into two meals. The CR monkey diet was then reduced by 10% per month until a 30% CR was achieved. Monkeys were maintained on either control (CON; two meals per day) or calorie restriction (CR; two meal reduced by 30% per day) for 1- 2 years. The animal groups are described below (Table 4.1).

Table 4.1 Distribution of Old Peri-Menopausal Rhesus Monkeys. Old control (OCON; 17-24 years) and old CR (OCR; 19-24 years) monkeys were identified for the study. Monkeys were maintained on a control (CON) or a moderate calorie restriction (CR; 30% reduced from CON feed levels) diet for 1-2 years.

	Age*	N
OCON	17-24	9
OCR	19-24	7

*Age in years at time of sampling.

Blood Collection and Baseline Hormone Analyses:

Monkeys were monitored for onset of menses (designated Menses d1). Serum was collected from the saphenous vein for 3 cycles (or approximately 90

days) following a spontaneous menses: daily during the follicular phase and every third day during the luteal phase. Circulating hormones were assayed for estradiol (E2) and progesterone (P4) by specific electrochemoluminescent assay using a Roche Elecsys 2010 assay instrument at the Endocrine Services Laboratory Core, ONPRC (Beaverton, OR). Luteinizing hormone (LH) was determined using a Leydig cell bioassay and follicle-stimulating hormone (FSH) was determined using an in-house radioimmunoassay (RIA). Hormone concentrations were validated against previous RIAs in this laboratory (Hess et al., 1981; Zelinski-Wooten et al., 1995).

E2 and P4 were retrospectively synchronized relative to the estimated mid-cycle LH peak (designated LH d0). This separates the follicular and luteal phases and allows analyses between comparable physiological states. Monkeys were assigned to one of two groups. Mean hormone levels were calculated for each group (OCON or OCR).

Inhibin B was determined using the commercially available Active Inhibin B kit (Diagnostic Systems Laboratory; Webster, TX). This ELISA was validated for use with rhesus monkey serum in our laboratory. Parallel displacement curves were obtained by comparing serially diluted (1:1 to 1:16) pooled rhesus monkey serum against known INHB concentrations. Recovery of known concentrations of unlabeled INHB added to pooled rhesus monkey serum was 100% with a coefficient of variation of 10%. Inter-assay coefficients of variation for two internal controls were 15% and 9%. The intra-assay coefficient of variation was always less than 10%.

Controlled Ovarian Stimulation

In a spontaneous menstrual cycle, Antide (Ares Serono; 0.5 mg/kg BW, sc, at 0800h) was administered on d1-d9 to inhibit endogenous gonadotropin production and recombinant human FSH (r-hFSH; (Gonal-F; Ares Serono; 30 IU intramuscularly at 0800 and 1700)) was administered on d1-d6 followed by r-hFSH + r-h luteinizing hormone (Lahdi; Ares Serono; im, at 0800) on d6-d9 to stimulate the production of multiple preovulatory follicles. This was followed by an injection of h-chorionic gonadotropin (Serono; 1000 IU, im) on d10 or d11 to induce ovulatory maturation.

Response to COS was determined by peak E2 levels and ultrasound visualization of follicles. Monkeys were classified as Responder (R), Poor Responder (PR) or Non Responder (NR) based on the predetermined criteria (Table 4.2).

Table 4.2 Controlled Ovarian Stimulation (COS) criteria for classifying a responder (R), poor responder (PR) or a non responder (NR). Criteria for peak E2 and number and size of follicles described below.

Hormone Criteria	Ultrasound Criteria	Outcome
peak E2 > 1000 pg/mL	> 3 follicles of 4 mm diameter / ovary	R
peak E2 < 600 pg/mL	> 3 follicles of 4 mm diameter / ovary	PR
peak E2 < 600 pg/mL	< 3 follicles of 4 mm diameter / ovary	NR

Follicle Aspiration and Embryo Culture

Transabdominal ultrasonography, using an HDI 1000 system with a C8-5 scanhead (ATL, Bothell, Washington) was performed on d7 to confirm the presence of a minimum of 3 follicles of 4mm diameter on each ovary. Blood samples were also collected daily from the saphenous vein and E2 levels were

determined to indirectly assess follicular response. Follicular aspiration by laparoscopy was performed on anesthetized animals 27 h after r-hCG injection (Zelinski-Wooten et al., 1995).

Follicular aspirates containing LGCs were pelleted and purified over a Percoll gradient to remove red blood cell contamination, according to techniques outlines by Molskness et al. (Molskness et al., 1991). Only R and PR aspirates were processed, since NR monkeys did not respond to COS and were therefore not aspirated for LGCs or oocytes. LGCs were counted and cell viability was assessed using Trypan blue exclusion. Cells were placed on fibronectin-coated 48-well or 96 well plates and incubated in DMEM/F12 media containing insulin, transferring, selenium and LDL, with or without hCG in triplicate. Media was harvested after 24 hours and frozen at -20˚C until assayed for P4 and INHB. Triplicate values were averaged for each monkey prior to calculating group's means.

Oocytes were recovered and evaluated for nuclear maturity at collection and after 6 h in vitro. They were subsequently classified based on stage of nuclear maturity: presence of an intact germinal vesicle (GV), metaphase I (MI; absence of both a germinal vesicle and polar body), metaphase II (MII; absence of a germinal vesicle and presence of an extruded polar body in the perivitelline space) or atretic (presence of presence of fragmentation or vacuoles in ooplasm and/or aspherical shape).

The oocyte data for the young animals was unavailable; only oocytes collected from OCON-R and OCR-R monkeys were fertilized and monitored for

embryo development. Immature (GV and MI) oocytes were allowed to incubate to maturity (MII) and subsequently fertilized via intracytoplasmic spermatozoa injection (ICSI). Mature oocytes were inseminated in vitro 6 hr after collection with 5 x 10^6 motile activated sperm (Hibbert et al., 1996; Zelinski-Wooten et al., 1995). Oocytes were initially classified according to the number of pronuclei visible on day 1 (16 h after insemination) as follows: 1) fertilized embryos (2 pronuclei and 2 polar bodies in the perivitelline space); 2) polyspermic embryos (with 3 or more pronuclei); and 3) unfertilized oocytes (with $\leq$1 pronuclei, oocytes remaining as MI or MII). On day 2, embryos were graded according to evenness of blastomeres, fragmentation and presence of cellular debris from perfectly symmetrical embryos with no fragmentation to embryos having one intact blastomeres with gross fragmentation or being totally degenerate (Hardy et al., 1989). All grading was performed by an observers who were blind to group identification. In addition embryos that did not cleave into 2-cell embryos with normal morphology were classified as having abnormal cleavage. Subsequently 2- to 4-cell embryos produced by IVF were co-cultured on Buffalo Rat Liver (BRL) cells in CMRL-1066 culture medium containing 10% fetal calf serum; media was changed on alternate days. This culture system supports development of IVF-produced macaque embryos to the hatched blastocyst stage (Weston et al., 1996; Zhang et al., 1994). Using an Olympus inverted microscope embryos were charted daily for development from 4-cell to 32-cell, morula (M), blastocyst (B), expanded (XB) and hatched blastocyst (HB) stages or through 10 days in culture (Weston et al., 1996).

Statistical Analyses:

Data are expressed as mean ± SEM for each parameter measured in each group. Standard descriptive statistics were performed. Log transformation of hormones was necessary and homogeneity of variances and normality of residuals was tested. Hormones were analyzed by 2-way ANOVA (repeated measures as necessary), followed by Tukey's for post-hoc comparisons (SAS; Cary, NC) and significant differences were established at $p < 0.05$.

RESULTS:

Baseline Hormone Analyses

6 of 9 OCON (Figure 4.1A) and 5 of 7 OCR (Figure 4.1B) monkeys exhibited normal, cycling hormone patterns: mid-cycle E2 peak followed by extended luteal phase levels of P4 (> 1 ng/mL). The remaining animals could not be aligned relative to the LH peak due to onset of peri-menopause and irregular menstrual cycles. Cycling monkeys in both CON and CR groups show comparable hormone profiles.

Controlled Ovarian Stimulation (COS)

Figure 4.2A shows the COS protocol for follicle stimulation following onset of menses (d1). Figure 4.2B depicts a representative E2 profile for a responder during a COS and the corresponding ultrasound taken on day 7.

Peak E2 levels were used to indirectly evaluate ovarian response to COS. Mean ± standard error peak E2 levels are shown in Figure 4.3. Responders (R) had significantly elevated peak E2 levels as compared to bother poor responders (PR) and non responders (NR). There were no differences between CON or CR groups.

Table 4.3 shows the mean peak E2 for each R, PR and NR groups as well as age distributions. The average age of OCON-PR was greater ($p < 0.05$) than that of OCON-PR.

Follicle Aspiration and Embryo Culture

Media collected after 24 hours LGC culture with or without hCG were measured for P4 and INHB. Figure 4.4A shows the mean P4 media levels in OCON and OCR monkeys with or without hCG. P4 significantly increased ($p < 0.05$) in hCG treated cells as compared to no hCG, however there were no diet effects. Figure 4.4B shows the mean INHB media levels in OCON and OCR monkey with or without hCG. There was no response to hCG treatment and no differences between OCON or OCR.

Following follicle aspiration, oocytes were separated from the follicular fluid and counted. There was no difference between OCON and OCR in the number of eggs collected (Figure 4.5A). Figure 4.5B shows the stage of nuclear maturity of the collected oocytes just after aspiration. There were no differences between OCON and OCR at any stage.

Following ICSI, zygotes were monitored for evidence of fertilization (presence of 2 polar bodies). Figure 4.6A shows the percentage of oocytes collected from OCON-R and OCR-R monkeys, successfully fertilized by ICSI. There were no differences between OCON-R and OCR-R.

Figure 4.7 shows the embryonic development of fertilized oocytes collected from OCON-R and OCR-R monkeys. OCR-R embryos had improved ($p<0.05$) embryo development, as compared to OCON-R embryos over all stages. Furthermore, only embryos from OCR oocytes developed past the blastocyst stage.

Figure 4.8 shows the rate of embryonic development in OCON and OCR. OCON embryos arrest at the blastocyst stage and take longer to develop than in OCR. OCR embryos continue on to expanded and hatched blastocyst stages.

DISCUSSION

The calorie restriction paradigm has long been recognized as an effective nutritional intervention for life extension in many species (Mattison et al., 2003; McCay et al., 1935; Roth et al., 1999; Smith et al., 2004). Although the effects of CR on extending reproductive lifespan in rodents are mixed (Holehan and Merry, 1985c; Merry and Holehan, 1979; Nelson et al., 1985), the potential for developing a means for delaying menopause would be extremely beneficial. This study is not only the first to evaluate the effects of short-term CR in older, peri-menopausal rhesus monkeys and their response to follicle stimulation; but

also the first to demonstrate that embryonic development is actually improved in monkeys maintained on the CR diet.

Although there were relatively few differences in diet with regard to ovarian hormone response to COS, number of oocytes retrieved and level of nuclear maturity, there were surprising differences between diet groups in embryo development in old monkeys. Overall embryo development was better in OCR as compared to OCON. Finally, and perhaps most interestingly, only embryos from OCR monkeys progressed beyond the blastocyst stage. There were no OCON embryos observed at expanded blastocyst or hatched blastocyst stages.

Careful study of the E2 and P4 dynamics within a spontaneous menstrual cycle established baseline hormone data. These data corroborated previous findings that menstrual cycles appear normal in monkeys maintained for short-term (1-2 years) CR diet with some irregularity expected with age. The reproductive axis can be very sensitive to changes in nutrition and energy balance (Harber, 2000; Harber, 2004; Taylor et al., 1999; Williams et al., 2001), therefore it was important to ascertain that monkeys were in fact cycling with some normality (i.e. CR similar to CON) prior to follicle stimulation.

Historically, it has been believed that female vertebrates acquire 100% of their primordial follicles at or around the time of birth. This doctrine has recently been brought into question by Johnson and colleagues (Johnson et al., 2004) who demonstrated that ovarian follicular renewal may be possible in mice. Regardless of the process, by menopause the ovary is almost completely devoid

of follicles. Throughout the reproductive lifespan of any female, the number of follicles that become atretic is much greater than the number of follicles that actually proceed through to ovulation.

With age, the likelihood of pregnancy success, even with artificial reproductive technologies, is greatly reduced (Broekmans et al., 2004; Broekmans and Klinkert, 2004). This brings into question the quality of oocytes remaining in the ovary at peri-menopause. The technique of aspirating follicles following gonadotropin stimulation may result in the retrieval of atretic or "bad" eggs (Balmaceda et al., 1994; Lim and Tsakok, 1997; Navot et al., 1991; Serna and Garcia-Velasco, 2005). Thus age and ovarian reserve are both important factors in evaluating response to gonadotropin treatment (Keck et al., 2005).

We investigated the effects of CR on ovarian response to follicle stimulation and oocyte competence and embryogenesis *in vitro* in old rhesus monkeys. Furthermore, we were able to monitor response to the COS both indirectly and directly, using peak E2 and ultrasound visualization of follicles. It was interesting to note that monkeys naturally divided into three distinct groups (responders, poor responders and non responders). Furthermore, within these groups, although there were no differences in peak E2 between OCON and OCR, the OCON-PRs were older than OCR-PRs. This suggests that the CR treatment permitted older monkeys to continue to respond to the COS at older ages as compared to CON monkeys.

COS-stimulated follicles yield mature oocytes capable of fertilization following insemination *in vitro* as well as subsequent development to pre-

implantation embryonic stages in vitro. The ovarian follicle is lined with granulosa and theca cells that respond to changes in the hormonal milieu. Furthermore, they are themselves hormonally active and contribute to oocyte development. As expected, LGCs in both groups responded to hCG with an increased production of P4; however, there were no dietary differences. Furthermore, there was no change or difference in diet, in the levels of INHB produced by the LGCs in culture. We were unable to assess granulosa cells prior to hCG injection and luteinization, and there may be some effects that are unaccounted for in this regard. However, improved embryo development seen in our results can also due to effects of CR on the oocyte itself. Clavero, et al. (Clavero et al., 2003) demonstrated that there was no correlation between oocyte maturity or fertilizability by ICSI in the percentage of apoptosis in granulosa cells collected by follicle stimulation. Numerous other factors may be differentially regulated in CR monkeys that we have not addressed and have been shown to play a role in oocyte and embryo development including c-kit ligand, bone morphogenic proteins (BMPs), insulin like growth factor (IGF), vascular endothelial growth factor (VEGF), leptin or nitric oxide (Barroso et al., 1999; Driancourt et al., 2000; Goya et al., 2002; Hutt et al., 2006; Klein et al., 2000; Thomas et al., 2005).

The large cytoplasmic contribution of the oocyte to the embryo may contribute to the differences we observed in embryo development with CR. Within the cytoplasm of the egg are mitochondrial DNA as well as a multitude of cytoplasmic proteins, such as cytoskeletal elements, enzymes, energy stores,

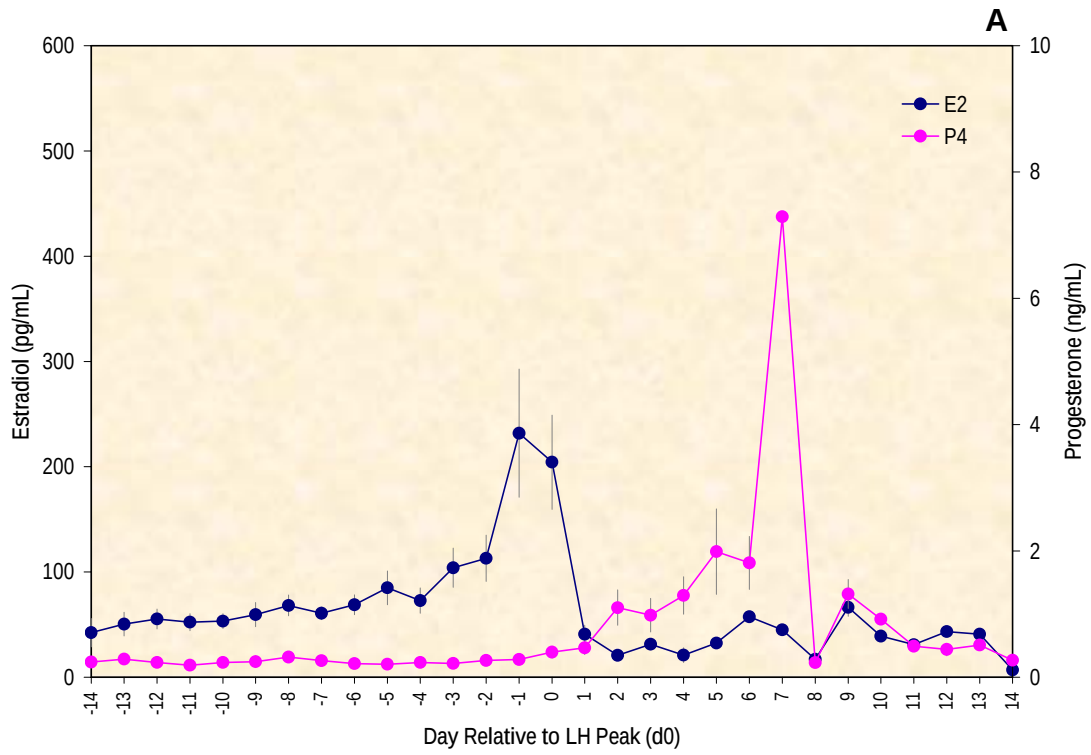
cell cycle regulatory proteins (Jansen and Burton, 2004; Krey et al., 2001; May-Panloup et al., 2004). Within human assisted reproduction, it is believed that abnormalities in the oocyte cytoplasm contribute to low pregnancy rates, even with supplemental protocols (Fulka et al., 2001; Fulka et al., 2005). Mitochondrial dysfunction in the oocyte has been suggested as one of the causes of embryonic developmental retardation and arrest (Nagai et al., 2004).

Although scientists are still investigating the phenomenon of life extension via CR, one hypothesis is that CR reduces the extent of oxidative damage caused by free radicals (Merry, 2004). Mitochondrial damage may occur as a result of excessive reactive oxygen species and may in turn generate damage oxyradicals at an even greater rate. Caspase cascades, followed by apoptosis are common responses to mitochondrial damage. APAF-1 binding to free cytochrome c (mitochondrial protein associated with oxidative phosphorylation and released as a result of cellular damage) can trigger caspase aggregation and apoptosis. Free radical formation is inherent to mitochondrial function and therefore, no system is exempt from its effects. Therefore, reduced levels of free radicals, via CR, may have possible benefits to the developing oocyte (Agarwal et al., 2005).

We conclude that CR may prolong ovarian responsiveness to exogenous gonadotropins and support oocyte nuclear maturation, fertilization and embryonic development *in vitro* in old female rhesus monkeys. Therefore, short-term (1-2 years) exposure to moderate CR in old female rhesus monkeys has beneficial effects on ovarian function.

ACKNOWLEDGMENTS

This research was supported by NIH AG21382-01 and OHSU RR00163. We would like to thank the members of the Oregon National Primate Research Center (ONPRC): Diana Takahashi, Maralee Lawson, Dr. David Hess, Dr. Frank Koegler, Dr. Francis Pau, Dr. John Fanton, Dr. Ted Hobbs, Megan Martin, Lindsay Pranger and Olivia Thoene.


A
E2
P4
Estradiol (pg/mL)
Progesterone (ng/mL)
Day Relative to LH Peak (d0)

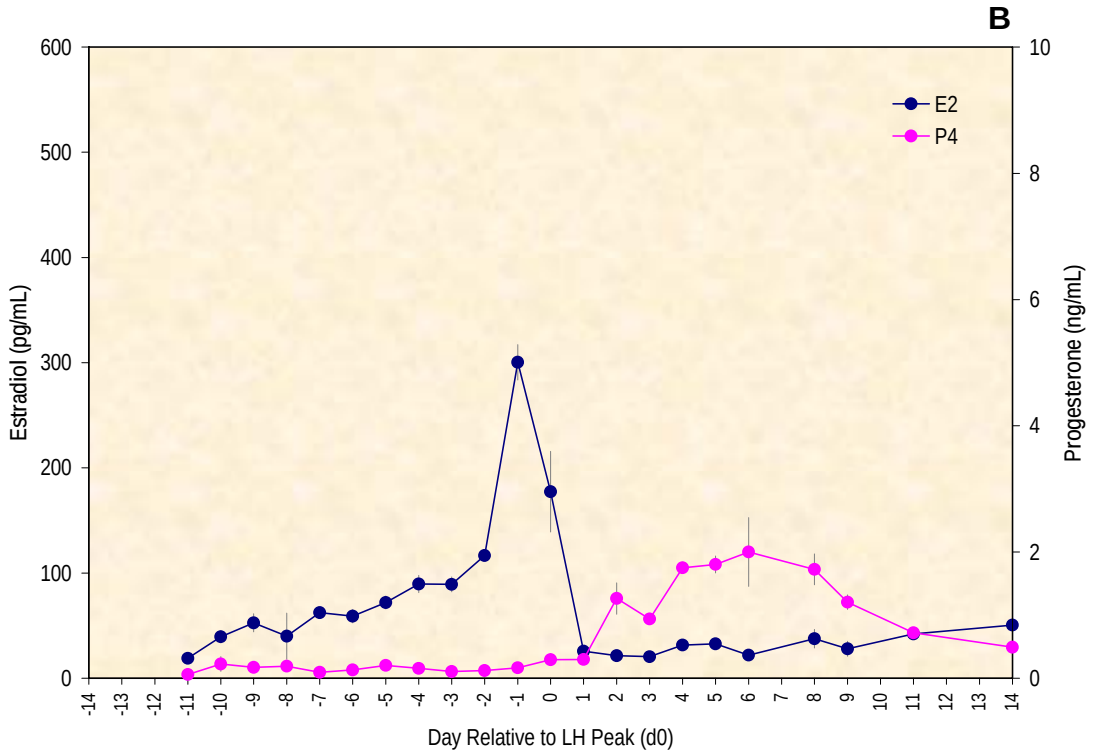
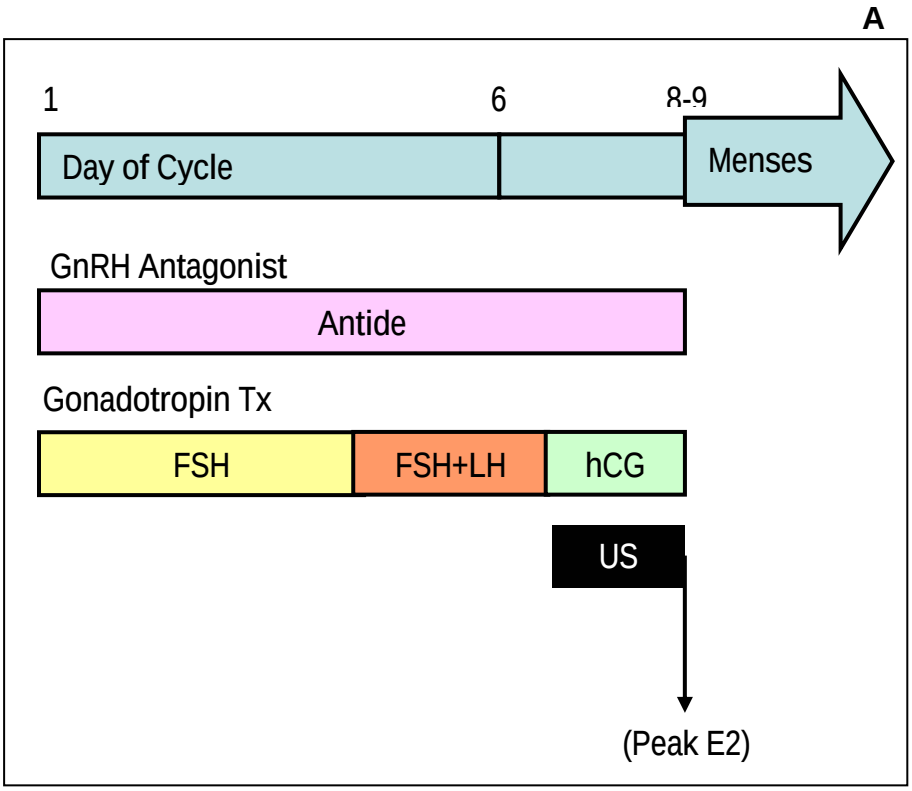

B
E2
P4
Estradiol (pg/mL)
Progesterone (ng/mL)
Day Relative to LH Peak (d0)

Figure 4.1 Hormone Profiles for OCON and OCR. Graphs depict mean hormone profiles in a spontaneous menstrual cycle. A) OCON (n=6), B) OCR (n=5) are graphed relative to the LH peak (not shown; d=0). E2 levels are pink, P4 levels are blue. E2 peaks may be observed prior to luteal levels of P4. Blood samples were collected from the saphenous vein daily during the follicular phase and every third day during the luteal phase during a spontaneous menstrual cycle.


A
1
6
8-9
Day of Cycle
Menses
GnRH Antagonist
Antide
Gonadotropin Tx
FSH
FSH+LH
hCG
US
(Peak E2)

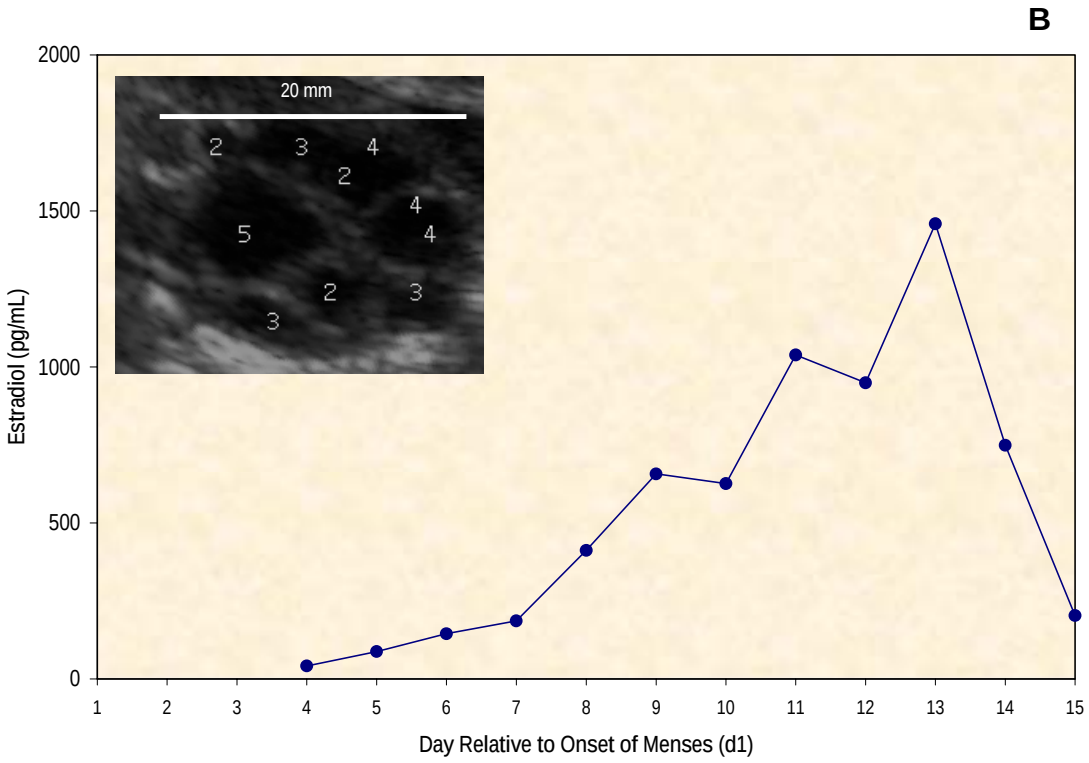

B
20 mm
Estradiol (pg/mL)
2000
1500
1000
500
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Day Relative to Onset of Menses (d1)

Figure 4.2. Controlled ovarian stimulation (COS) in rhesus monkeys. A) COS protocol: administration of Antide (to suppress endogenous hormone production), h-rFSH, h-rLH, hCG to induce ovulatory maturation of follicles and laparascopic follicle aspiration (FA). B) Representative Responder E2 profile in response to COS and ultrasound of a COS-stimulated ovary.

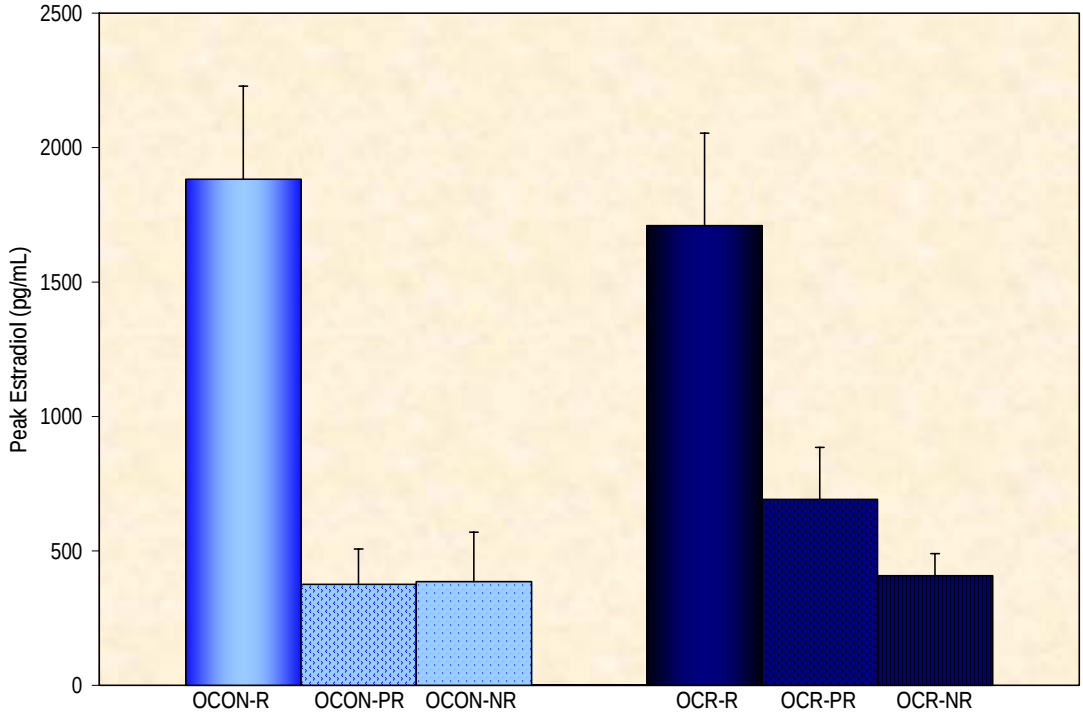
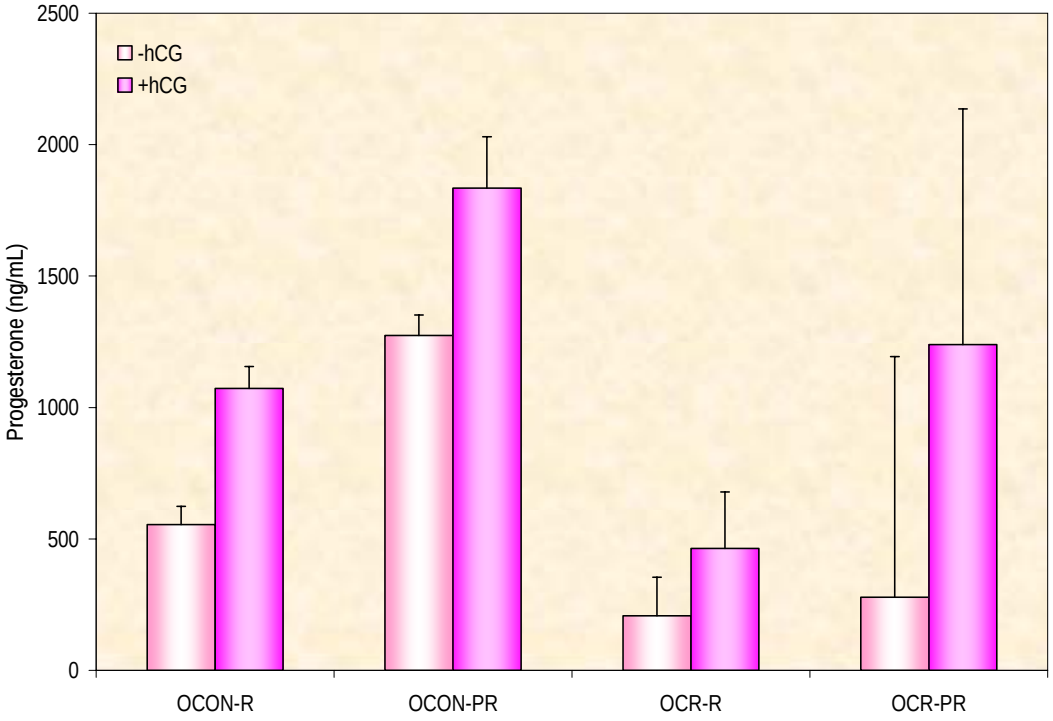


Figure 4.3 Peak E2 levels in response to COS. OCON are light blue bars, OCR are dark blue bars. Mean peak E2 levels are shown for Responders (OCON-R, OCR-R), Poor Responders (OCON-PR, OCR-PR) and Non Responders (OCON-NR, OCR-NR). Peak E2 in R monkeys was significantly higher ($p < 0.05$) than in PR or NR. No differences were found between OCON or OCR in any groups.

Table 4.3. Age distribution and peak E2 following COS in OCON and OCR monkeys. Ages were similar for COS response in OCON-R and OCR-R as well as for OCON-NR and OCR-NR, however OCON-PR monkeys were significantly younger than OCR-PR monkeys.

Cohort	N	Peak E2	Age
OCON-R	4	1883 ± 346	20 ± 1.0
OCON-PR	2	376 ±132	21 ± 2.0
OCON-NR	3	386 ± 183	24 ± 0.3
OCR-R	3	1710 ± 344	20 ± 0.9
OCR-PR	2	692 ± 194	25 ± 0.5
OCR-NR	2	408 ± 82	24 ± 0.0


2500
2000
1500
1000
500
0
Progesterone (ng/mL)
-hCG
+hCG
OCON-R
OCON-PR
OCR-R
OCR-PR

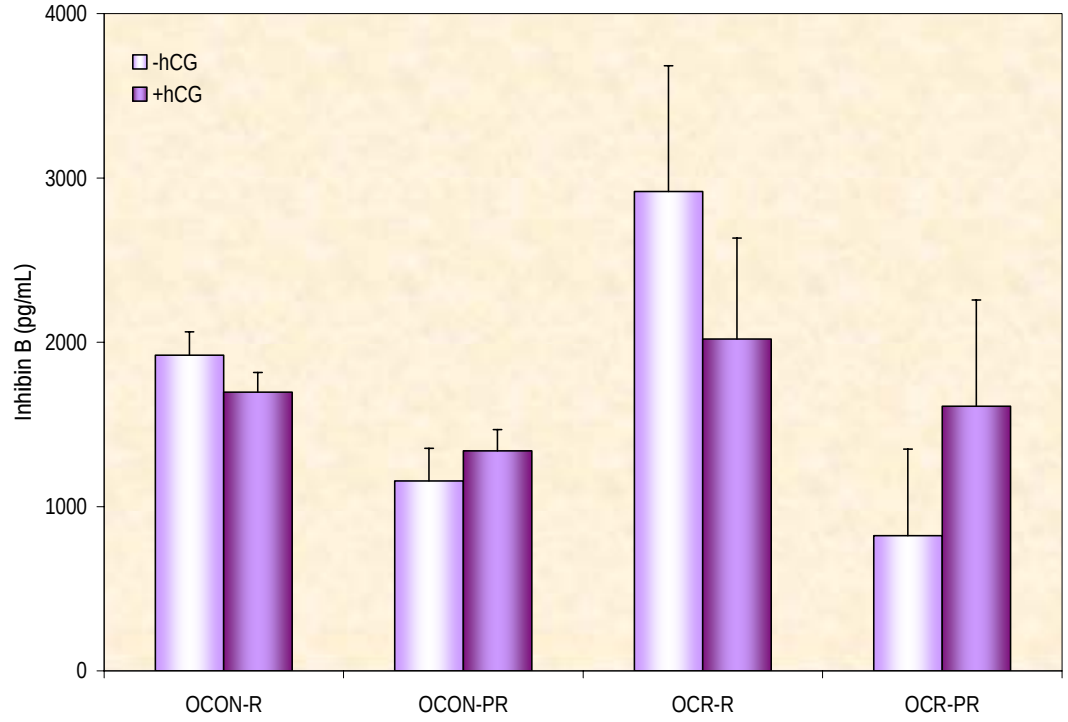
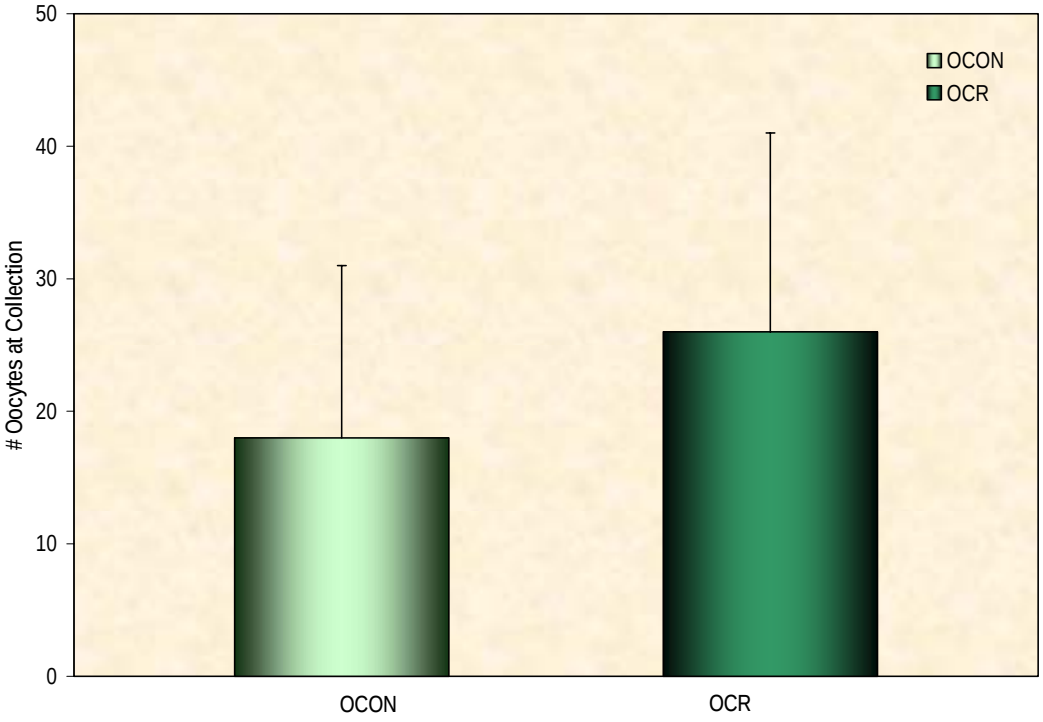

4000
3000
2000
1000
0
Inhibin B (pg/mL)
-hCG
+hCG
OCON-R
OCON-PR
OCR-R
OCR-PR

Figure 4.4 Media hormone levels in LGC culture. A) This graph depicts mean P4 levels in media with or without hCG. hCG-treated cells had significantly higher ($p<0.05$) levels of P4, however no diet effects were found. B) This graph shows INHB levels in LGC cultures with or without hCG. No differences were found either with hCG treatment or diet. Only OCON-R, OCON-PR, OCR-R and OCR-PR were included in the above analyses. OCON-NR and OCR-NR did not respond to COS and were therefore not aspirated for LGCs or oocytes.


50
40
30
20
10
0
Oocytes at Collection
OCON
OCR
OCON
OCR

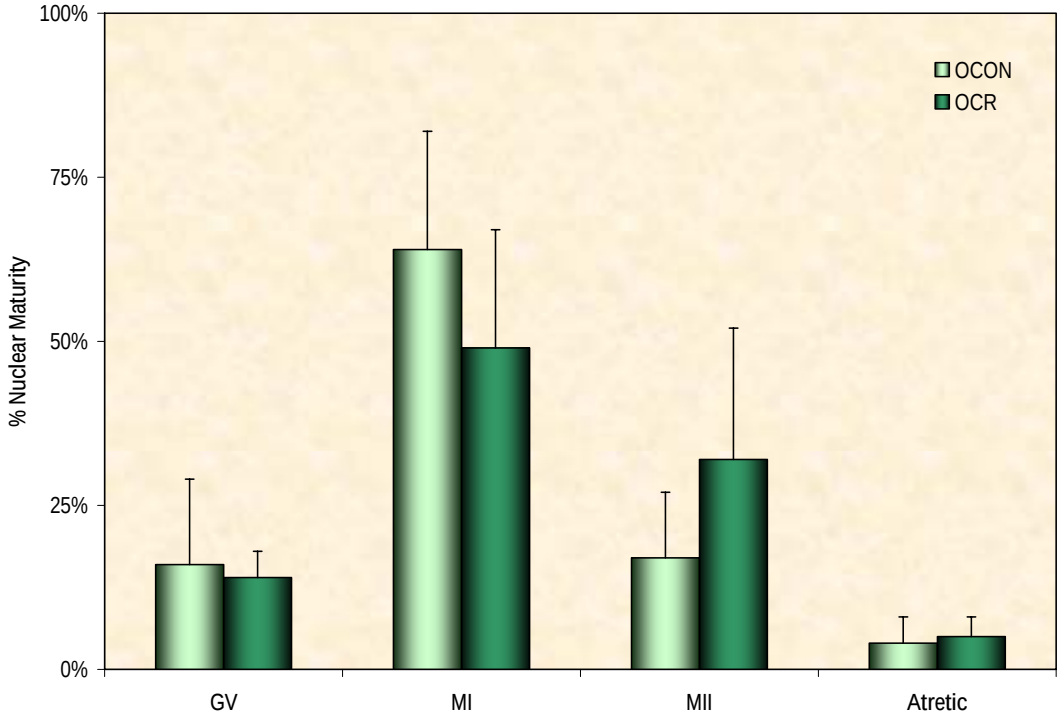

100%
75%
50%
25%
0%
% Nuclear Maturity
OCON
OCR
GV
MI
MII
Atretic

Figure 4.5 Number of oocytes collected and stage of nuclear maturation. Oocyte data were collected from OCON (R and PR) and OCR (R and PR). A) This graph shows the mean number of oocytes collected via laparascopic follicle aspiration. No differences were detected between OCON and OCR. B) This graph shows the distribution of oocyte nuclear maturity at the time of collection. Nuclear maturity was assessed by: presence of a germinal vesicle (GV), absence of a polar body (MI), presence of a polar body (MII) or atretic. No differences were measured between OCON and OCR at any stage.

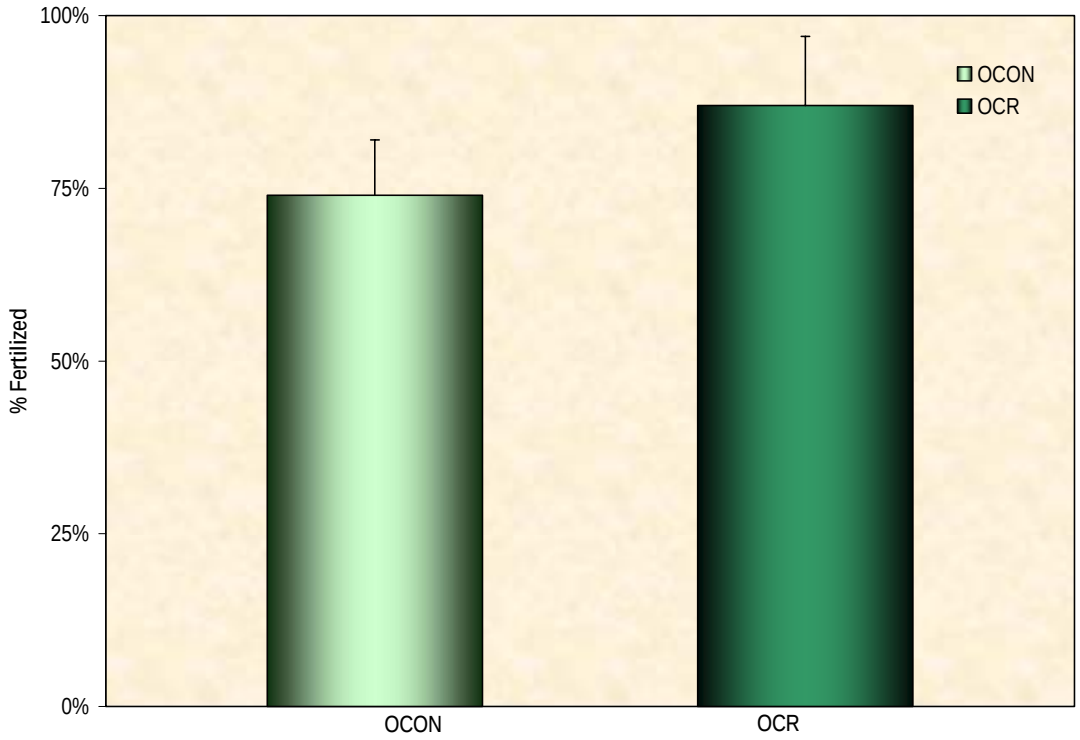


Figure 4.6 Percentage of successful fertilization. This graph shows the percentage of oocytes successfully fertilized (presence of 2 polar bodies) via ICSI. No differences were found between OCON and OCR.

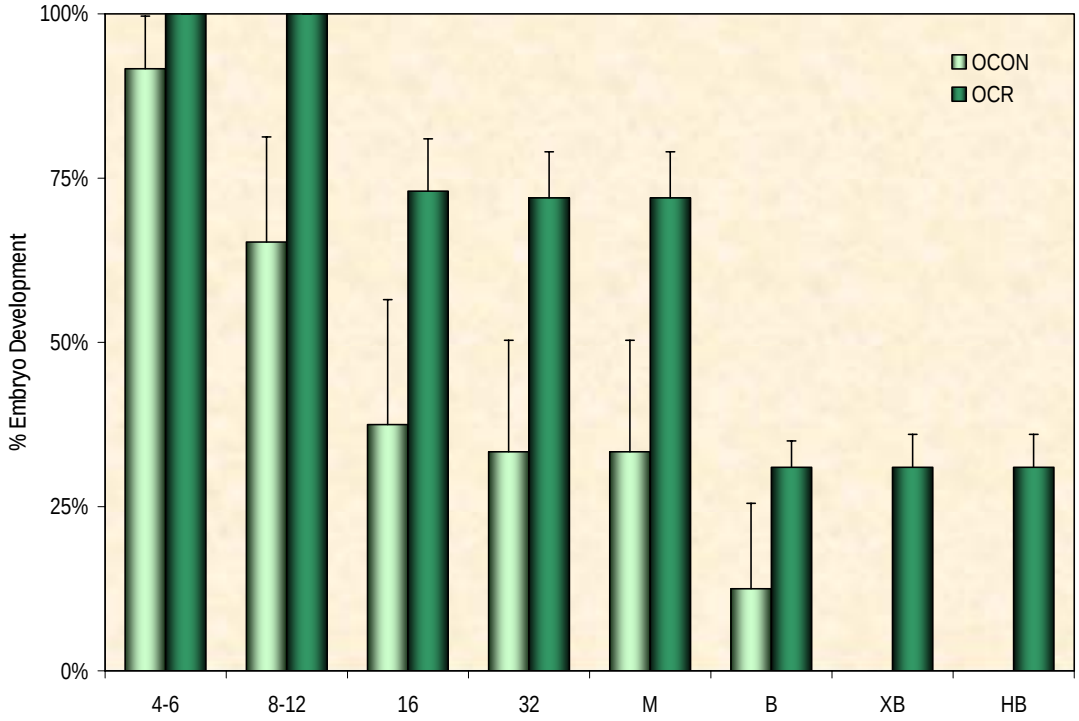


Figure 4.7 Embryo development. This graph depicts embryo development using *in vitro* culture conditions following fertilization of oocytes collected from OCON-R and OCR-R monkeys. Percent embryos progressing to each of the following stages: 4-6 cells, 8-12 cells, 16 cells, 32 cells, morula (M), blastocyst (B), expanded blastocyst (XB) and hatched blastocyst (HB) were determined. Embryos from oocytes collected in OCR monkeys had improved ($p < 0.05$) embryonic development as compared to OCON.

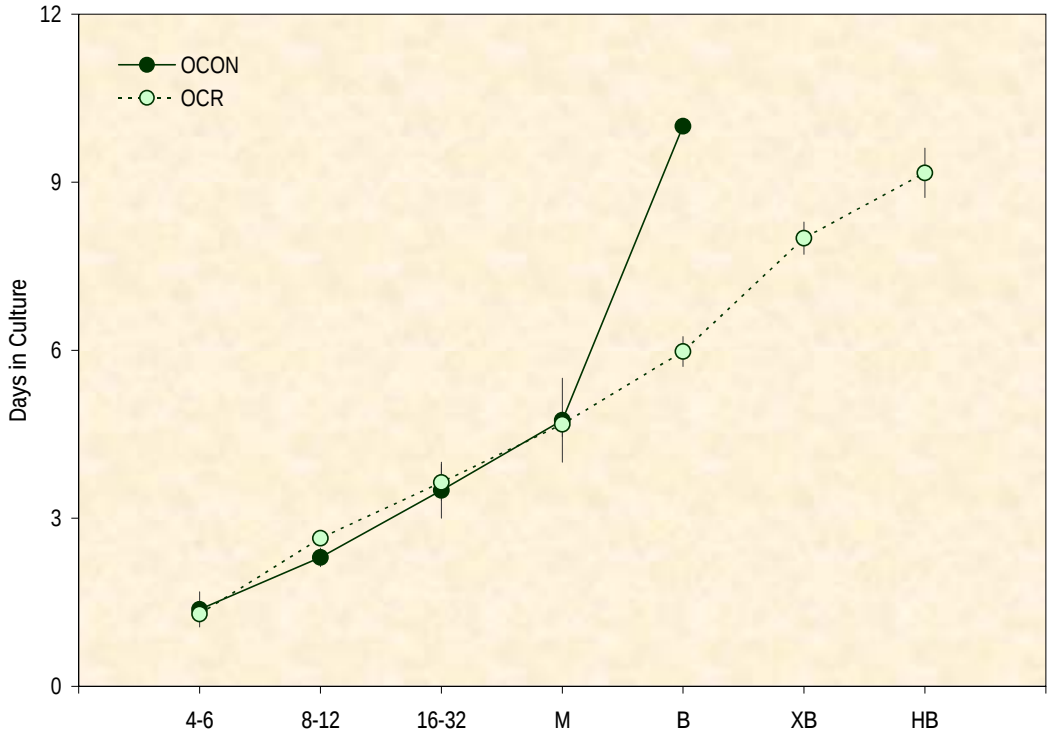


Figure 4.8 Rates of Embryo Development. Embryo development rates from oocytes collected from old CON (OCON) and old CR (OCR) monkeys. Stages of embryonic development were assessed at 4-6 cells (4-6), 8-12 cells (8-12), 16-32 cells (16-32), morula (M), blastocyst (B), expanded blastocyst (XB) and hatched blastocyst (HB). OCON embryos arrested at the blastocyst stage and took longer to develop in culture. OCR embryos continued to develop to expanded and hatched blastocyst stages.

CHAPTER 5

EFFECTS OF CALORIE RESTRICTION ON RHESUS MONKEY GENE EXPRESSION IN LUTEINIZING GRANULOSA CELLS

INTRODUCTION

Calorie restriction (CR) has been proven to increase longevity in a number of species, including *C. elegans*, *D. melanogaster*, rats, mice and limited data in primates (Lane et al., 1996; Lane et al., 1997; Lane et al., 2000; Le Bourg, 2005; Smith et al., 2004). The mechanism of action of CR is not fully understood, however, researchers have demonstrated reduced damage incurred by free radical production via oxidative stress (Merry, 2004).

Data on the effects of CR on reproduction are mixed and titration of level of CR and species response is necessary. Data from rats and mice are inconsistent with regard to CR effect on reproduction. In mice, CR disrupts normal cycling; however, resumption of normal reproductive function occurs with re-feeding (Nelson et al., 1985; Nelson et al., 1995). Furthermore, the onset of ovarian decline was subsequently delayed as well. In rats, CR has been proven to increase reproductive longevity without disrupting normal cycling (Holehan and Merry, 1985a; Merry and Holehan, 1985). Preliminary data in rhesus monkeys (ages 7-27 years) suggested no adverse effects in long-term CR in rhesus monkeys with regard to reproductive hormones measured on menstrual day 5 (Black et al., 2001) .

There has been only limited research in granulosa cell microarrays. One study investigated the differences in gene expression in granulosa cells stimulated with or without forskolin, however these cells were collected from women 26-32 years, without regard for relative ovarian reserve or menstrual cyclicity. (Sasson et al., 2004). Another recent study reported gene expression profiles in granulosa cells collected from women suffering from polycystic ovarian syndrome (PCOS) as compared to normal ovaries (Jansen et al., 2004).

DNA microarray analyses of luteinizing granulosa cells by Chin et al. (Chin et al., 2002) demonstrated differences in gene expression between women with normal or decreased ovarian reserve. Diminished ovarian reserve was determined based on day 3 FSH and peak serum E_2 levels and number of oocytes retrieved following an ovarian stimulation protocol. Chin, and colleagues (Chin et al., 2002) admit the difficulty in interpreting these data, given small sample sizes, differential responses within groups, and inconsistent gonadotropin stimulation of subjects. It is still of great interest, however, to note that there were changes in a few specific genes. Although inconclusive, their data provide a basis for identifying specific gene targets that vary within the ovary during aging that will be useful for future research on ovarian aging and menopause in women.

This chapter is based on preliminary findings evaluating the use of microarrays in evaluating gene expression changes in LGCs collected from young and old rhesus monkeys maintained on short-term calorie restriction.

MATERIALS AND METHODS

Animals and Diet

Young and old female rhesus monkeys were identified for this study. Animals were offered food and water ad libitum. Average daily food consumption was determined for each monkey and divided into two meals per day. Feed allotment in calorie restricted (CR) monkeys was then reduced by 10% per month, until 30% restriction was achieved. Distribution of animals is indicated in Table 5.1.

Table 5.1 Distribution of Animals. Young control (YCON), young CR (YCR), old control (OCON) and old CR (OCR) were identified for this study. Animal numbers within each group are indicated below.

Group	N
YCON	5
YCR	5
OCON	9
OCR	8

Controlled Ovarian Stimulation (COS) and Follicle Aspiration

Monkeys were maintained on short-term CR for 1-2 years and monitored for onset of menses (d1). A controlled ovarian stimulation (COS) was performed to stimulated the production of multiple pre-ovulatory follicles. Monkeys received Antide (Ares Serono; 0.5 mg/kg BW, subcutaneous, at 0800h) from d1-d9. They received 30 IU recombinant human follicle-stimulating hormone (r-hFSH; Gonal-F; Ares Serono; intramuscular, twice daily) from d1-d6; followed by 30 IU r-hFSH + r-h luteinizing hormone (r-hLH; Lahdi; Ares Serono; Rockland, MA); im, once daily) on days d7-d9. Trans-abdominal ultrasonography was performed on day 7 of the protocol. When at least 3 follicles of > 4 mm diameter were visualized on

each ovary, r-h chorionic gonadotropin (r-hCG; Serono; Rockland, MA; 1000 IU) was administered the next day to mimic the LH surge and induce ovulatory maturation of follicles. Follicle aspiration via laparoscopy was performed 27 h following the r-hCG injection. If ultrasound follicle criteria were not met, the COS was canceled and no follicle aspiration was performed. Follicle stimulation and aspirations were repeated once, because cell number and consequently RNA yield was expected to be low.

Peripheral blood mononuclear cells (PBMCs) collected from two unrelated control female rhesus monkeys were graciously donated by Dr. Janko Nikolich-Zugich and used as a pooled reference control.

RNA Extraction, Amplification and Labeling

Follicular aspirates were collected and oocytes were identified and removed from the aspirates. The remaining aspirates containing luteinizing granulosa cells were pelleted and purified over a Percoll gradient to remove red blood cell contamination, according to techniques outlined by Molskness and colleagues (Molskness et al., 1991). Cells were counted and cell viability was assessed using Trypan blue exclusion. Finally cells were stored in 1 mL TRIZOL Reagent (Invitrogen; Carlsbad, CA) and frozen at -80°C.

Luteinizing granulosa cells were extracted following the protocol outlined by Invitrogen for use with TRIZOL Reagent. Briefly, cells were thawed and centrifuged to separate the aqueous and organic phases. The aqueous layer was then processed to isolate pure RNA. RNA concentration and quality was determined using an Agilent 2100 Bioanalyzer. For monkeys with more than one

follicle stimulation and LGC aspiration, the sample with better quality and yield was chosen for microarray analysis. RNA samples were then converted to cyanine5 (Cy5)- and Cy3- labeled complementary RNA (cRNA) using the Agilent low RNA input fluorescent linear amplification kit. Samples were subsequently purified using the Agilent cRNA cleanup kit and the concentration and dye incorporation was determined using a Nano drop spectrophotometer.

Microarrays

Twenty Agilent whole human genome oligo microarray kits were generously donated by the National Institute on Aging for this experiment. This array has 44,000 60-mer oligonucleotides representing approximately 41,000 genes and transcripts. Rhesus monkeys have a high similarity to humans and we anticipated no problems using a cross-species microarray. Cy-5 and Cy3-labeled samples were hybridized and washed according to manufacturer's recommendations. They were then scanned using an Agilent microarray scanner (Model G2565BA). The relative fluorescent intensities were determined and the Agilent feature extraction software was used for statistical analyses. Fluorescence data was then imported in JMP statistical software (Cary, NC) for further analysis.

Data were checked for normality, symmetry, range of the data and low standard deviation as criteria. Principal component analyses were then performed, followed by replicate correlation analyses to determine if the data could be used as biological replicates.

Genes were classified based on up-regulated or down-regulated gene changes. Statistical differences were noted at $p < 0.05$. Gene changes were based on z-ratios and only changes greater than 1.5 or less than -1.5 were included. Additionally, only genes with average intensities greater than 0 were used; this excluded high fold changes that result from intrinsically low values. Twelve comparisons were made between groups as follows: gene expression changes (up-regulation or down-regulation) in YCON versus YCR, YCON versus OCON, YCR versus OCR, OCON versus OCR, YCON versus OCR, and YCR versus OCON.

RESULTS

Controlled Ovarian Stimulation (COS) and Follicle Aspiration

All young monkeys responded to COS (twice) and follicle aspirations were performed to collect LGCs. Four of nine OCON and four of eight OCR responded to COS and were follicle aspirated at least once.

RNA Extraction, Amplification and Labeling

All LGC samples were extracted for RNA. A minimum of 50 ng high quality RNA was necessary to proceed with the amplification and labeling process. All LGCs collected from young monkeys had sufficient RNA to proceed; therefore a single sample (from the duplicate follicle aspirations) was subjectively chosen for each young female. Additionally, one YCON female and YCR female

had duplicate samples. Conversely, only four of five OCON and four of five OCR had sufficient RNA for RNA amplification and labeling (Table 5.2).

Table 5.2 Fluorescent-labeled cRNA for Hybridization. Young control (YCON), young CR (YCR), old control (OCON) and old CR (OCR) samples fluorescently labeled for hybridization. Animal numbers within each group are indicated below.

Group	N
YCON	5
YCR	5
OCON	4
OCR	4

Microarray Analyses

Data were normally distributed. Principal component analysis and replication correlation analyses were performed to confirm biological replication within groups.

Figure 5.1A shows the scatter plots for individual samples in the YCON group. YCON samples were designated YCN1-YCN5B. YCN5 was the selected female with two replicates (YCN5A and YCN5B) collected from both follicle aspirations. Based on the correlation coefficients and general shape of the scatter plots, we determined that four of the six samples (YCN3, YCN4, YCN5A and YCN5B) could reliably be used as replicates in our subsequent analyses.

Figure 5.1B shows the scatter plots for individual samples in the YCR group. YCR samples were designated YCR1-YCR5B. YCR 5 was the selected female with two replicates (YCR5A and YCR5B) collected from both follicle aspirations. Based on the correlation coefficients and general shape of the scatter plots, we determined that five of the six samples (YCR1, YCR2, YCR3, YCR4, YCR5A) could reliably be used as replicates in our subsequent analyses.

Figure 5.1C shows the scatter plots for individual samples in the OCON group. OCON samples were designated OCN1-OCN4. Based on the correlation coefficients and general shape of the scatter plots, we determined that two of the four samples (OCN2 and OCN3) could reliably be used as replicates in our subsequent analyses.

Figure 5.1D shows the scatter plots for individual samples in the OCR group. OCON samples were designated OCR1-OCR4. Based on the correlation coefficients and general shape of the scatter plots, we determined that two of the four samples (OCR1 and OCR2) could reliably be used as replicates in our subsequent analyses.

Table 5.3 shows the final distribution of samples following the biological replicate analysis. Biologically non-replicable samples were excluded from the analysis.

Table 5.3 Final distribution of samples after Biological Replicate Analysis. Young control (YCON), young CR (YCR), old control (OCON) and old CR (OCR) samples included in microarray analysis. Final animal numbers within each group are indicated below.

Group	N
YCON	4
YCR	5
OCON	2
OCR	2

Gene expression changes between groups were determined based on significance ($p < 0.05$), z-ratio and intensity. The relative up- or down-regulation of genes for each of the twelve comparisons is summarized in Table 5.4. Total gene changes as well as the subset of genes with known molecular functions are listed. Specific molecular functions were identified as prospective genes for

future real time polymerase chain reaction (RT-PCR) analysis. Specifically, apoptosis, mitochondrial, steroid or growth factor related functions were highlighted and listed in the following Tables.

Table 5.5 shows the specific gene function changes between YCON and YCR. Table 5.5A lists specific genes up-regulated in YCON as compared to YCR. Seven of 251 known genes were identified as potential genes of interest. Table 5.5B lists specific down-regulated in YCON as compared to YCR. Three of 87 known genes were identified as potential genes of interest.

Table 5.6 shows the specific gene function changes between YCON and YCR. Table 5.6A lists specific genes up-regulated in YCON as compared to YCR. Four of 203 known genes were identified as potential genes of interest. Table 5.6B lists specific down-regulated in YCON as compared to YCR. Seven of 118 known genes were identified as potential genes of interest.

Table 5.7 shows the specific gene function changes between YCR and OCR. Table 5.7A lists specific genes up-regulated in YCR as compared to OCR. Seven of 110 known genes were identified as potential genes of interest. Table 5.7B lists specific down-regulated in YCR as compared to OCR. 14 of 377 known genes were identified as potential genes of interest.

Table 5.8 shows the specific gene function changes between OCON and OCR. Table 5.8A lists specific genes up-regulated in OCON as compared to OCR. 6 of 137 known genes were identified as potential genes of interest. Table 5.8B lists specific down-regulated in OCON as compared to OCR. 17 of 382 known genes were identified as potential genes of interest.

Table 5.9 shows the specific gene function changes between YCON and OCR. Table 5.9A lists specific genes up-regulated in YCON as compared to OCR. 6 of 147 known genes were identified as potential genes of interest. Table 5.9B lists specific down-regulated in YCON as compared to OCR. 17 of 332 known genes were identified as potential genes of interest.

Table 5.10 shows the specific gene function changes between YCR and OCON. Table 5.10A lists specific genes up-regulated in YCR as compared to OCON. 5 of 158 known genes were identified as potential genes of interest. Table 5.10B lists specific down-regulated in YCR as compared to OCON. 6 of 174 known genes were identified as potential genes of interest.

DISCUSSION

These are preliminary data evaluating the use of microarrays to compare gene expression in LGCs collected from rhesus monkeys maintained on short-term CR. Based on the results of the microarray, we have been able to recommend responsive genes to pursue and validate by real-time polymerase chain reaction (RT-PCR).

Older rhesus monkeys exhibit a reduced response to COS and poor follicular development, despite treatment with exogenous gonadotropins. Consequently, our LGC yield, RNA quantity and quality were greatly reduced in the older monkeys. Once proceeding through the necessary RNA preparation and microarray analysis, our sample size for older monkeys had diminished greatly (OCON, n=2; OCR, n=2). Similar to the publication examining gene

expression changes in LGCs in humans, our data will be difficult to interpret due to small sample sizes (Chin et al., 2002).

The 12 comparisons evaluated in this chapter generated 4174 gene changes. Of those genes, 2476 had known molecular functions. We subjectively identified a handful of molecular functions with biological relevance with regard to our treatments (age and CR), namely apoptosis-, mitochondrial-, hormone-, and transcription-related functions. Our short-list of genes was then examined more carefully to further identify possibilities to pursue by RT-PCR, and is summarized in Table 5.11.

Two genes with apoptotic functions were identified by our microarray analysis were: Homo sapiens caspase 4, apoptosis-related cysteine protease (CASP4) and Homo sapiens Fas apoptotic inhibitory molecule (FAIM). These are potentially very interesting genes with regard to our research. Apoptosis and follicular atresia occurs with regularity in growing follicles and is considered to be a normal physiological process (Johnson, 2000a; Tilly et al., 1992). Conversely, the depletion of ovarian reserve also likely occurs via atresia and apoptosis. Apoptosis is a tightly regulated system in which cells undergo programmed cell death. A number of pro-apoptotic proteins, Bax, Bid and Bad participate in programmed cell death. In addition, caspases are proteases that split proteins at aspartate residues to break down cell structures and initiate a sequence of events that also participate in cell death.

Interestingly delayed ovarian aging has been documented in the *Bax*$^{-/-}$ mouse. Bax is a member of the Bcl-2 family of proteins which are considered to

be pivotal in the regulation of cell death pathways (Kim, 2005). The Bcl-2 family members are generally classified as either pro-apoptotic, such as Bax, Bid and Bad; or anti-apoptotic, such as Bcl-2 and Bcl-X_L (Kim, 2005). Kim and colleagues (Kim, 2005) have hypothesized that the *Bax* deficiency may have granted some protection to the granulosa cells and oocytes against apoptosis and consequently delayed ovarian senescence.

CASP4 is known to be involved in apoptosis, particularly in endoplasmic reticulum stress induced apoptosis (Hitomi et al., 2004). Our data suggest that CASP4 is up-regulated in OCON as compared to YCON and OCR groups. Furthermore, reports show that when CASP4 was restrained, apoptosis was arrested in sinonasal squamous carcinoma (Li et al., 2004).

FAIM is believed to be an antagonist of Fas-induced cell death (Sole et al., 2004). Fas is a cell-surface receptor protein that is believe to mediates apoptosis-inducing signals and participates in ovarian follicular atresia (Kaipia and Hsueh, 1997; Manabe et al., 2004; Sakamaki et al., 1997; Slot et al., 2006). Results from our microarray experiment indicate that FAIM in OCR is up-regulated as compared to YCON, YCR and OCON. This would fit in well with our hypothesis that CR may be mediated some apoptotic processes in the LGCs collected from older monkeys.

Reduced damage incurred by free radical production via oxidative stress has been suggested as mechanism of action of CR (Merry, 2004), therefore alterations in genes related to mitochondrial function may be involved in preserving ovarian or LGC function. One of the genes we identified as a

possibility for RT-PCR is Homo sapiens uncoupling protein 3 (UCP3). Our results show UCP3 to be down-regulated in OCR as compared to YCN, YCR and OCON. Uncoupling proteins (UCPs) are present in the inner mitochondrial membrane and UCP1 at least is involved in uncoupling oxidation from phosphorylation during respiration and thermogenesis (Mozo et al., 2005). UCP2 is believed to allows for proton leakage and may be involved in reduction of free radical formation in neuronal tissue (Bechmann et al., 2002; Conti et al., 2005). The role of UCP3, however, is less clear. Mozo and colleagues (Mozo et al., 2006) suggest that both UCP2 and UCP3 are not involved in respiration uncoupling in Chinese hamster ovary cells. Further work needs to be done both in understanding the role of UCP3 as confirming the changes we observed in our microarray experiment.

We also considered steroid binding as a potential molecular function of interest. We found gene expression changes in Homo sapiens estrogen-related receptor alpha (ESRRA). Our data suggest that ESSRA is up-regulated in OCR as compared to both YCON and YCR. ESRRA is an orphan receptor that is believed to regulate energy metabolism in concert with peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC1-α) (Schreiber et al., 2004; Schreiber et al., 2003; Willy et al., 2004). Furthermore, it is believed to regulate the expression of genes involved in oxidative phosphorylation and mitochondrial biogenesis and it has been suggested that ESRRA activity could be involved in changes that occur with the onset of metabolic disease, i.e. diabetes (Schreiber

et al., 2004; Willy et al., 2004). Again, without a full understanding of the functions of ESSRA, it is difficult to draw conclusions.

Finally, we identified two transcription factors of great interest: *Homo sapiens* forkhead box P1 (FOXP1) and *Homo sapiens* forkhead box O1A (FOXO1A). Forkhead transcription factors include Foxo1, Foxo4 and Foxo6 (Brenkman and Burgering, 2003). Foxo transcription factors may be considered analogous to the DAF-16 transcription factor (and thus part of the DAF-2 pathway) in the roundworm (*C.elegans*; (Kenyon et al., 1993). This is of considerable interest given exciting research which has shown that loss-of-function mutations in DAF-2 extends lifespan in the roundworm (Hosaka et al., 2004; Kenyon, 2001). The role of Foxo genes in mice is currently being explored, however it is clear that the individual genes (foxo1, foxo3a and foxo4) are functionally diverse (Burgering and Medema, 2003). *In vitro* data suggests a role for Foxo genes in cell cycle arrest, apoptosis and specific stress responses (Castrillon et al., 2003; Tran et al., 2003).

Of interest, is the *Foxo3a*$^{-/-}$ mouse which is a model for accelerated ovarian aging (Brenkman and Burgering, 2003; Castrillon et al., 2003). These mice appeared outwardly normal up to 48 weeks of age, with no differences in body weight, or increases in cancer, or mortality. With regard to reproduction, however, Castrillon et al. (Castrillon et al., 2003) reported sterility in these mice by 15 weeks of ages, despite normal sexual maturation (based on first litter). Histological analyses of ovaries indicated normal ovaries at postnatal day 3 (PD3), but by PD8, *Foxo3a*$^{-/-}$ ovaries were consistently enlarged. These mice

exhibited early follicular activation, maturation and atresia, consequently resulting in early depletion of ovarian reserve. By 20 weeks, *Foxo3a*$^{-/-}$ females demonstrated classic signs of hypogonadotropic (elevated FSH and LH), hypogonadism, typical of premature ovarian failure (Castrillon et al., 2003).

Our results show FOXP1 to be up-regulated in OCR as compared to both YCR and OCN. FOXP1 is a fairly newly identified transcription factor. It has been suggested as participating in macrophage differentiation (Jonsson and Peng, 2005) as well as a potential candidate tumor suppressor gene (Banham et al., 2001).

We found FOXO1A to be down-regulated in YCON as compared to both OCON and OCR. FOXO1A is believed to be involved in cell cycle regulation, blocking entry by regulating other proteins (Cunningham et al., 2004). It has been shown to induce cycle arrest and subsequently initiate apoptosis (Bois and Grosveld, 2003). Furthermore, it has been proposed as a putative tumor suppressor in specific cell lines (Bois et al., 2005).

This is the first attempt to analyze rhesus monkey LGCs by microarray, in aged or CR animals. Since the commencement of this study, however, a rhesus monkey microarray has become commercially available and may permit a more rigorous analysis in future studies. Our microarray had the advantage of representing 40,000 genes and may be potentially more informative, as we are still in a “search” mode.

Our results are preliminary, but very exciting. Validation by RT-PCR is highly recommended, especially with regard to the aforementioned genes. We

performed a subjective perusal of the gene changes and found many possible and intriguing options to pursue. There are, however, highly sophisticated programs that permit a more critical and in-depth selection of genes of interest and may be useful to consider in the future.

ACKNOWLEDGMENTS

This research was support by NIH AG21382-01 and OHSU RR00163. We would like to thank the National Institute on Aging for its generous donation of microarrays, as well as the assistance of Dr. Kevin Becker, William Wood and Dr. Vinayakumar Prabhu. Additionally, we would like to thank the staff at the Oregon National Primate Research Center (ONPRC): Diana Takahashi, Maralee Lawson, Dr. David Hess, Dr. Frank Koegler, Dr. Francis Pau, Dr. John Fanton, Dr. Ted Hobbs, Megan Martin, Lindsay Pranger and Olivia Thoene.

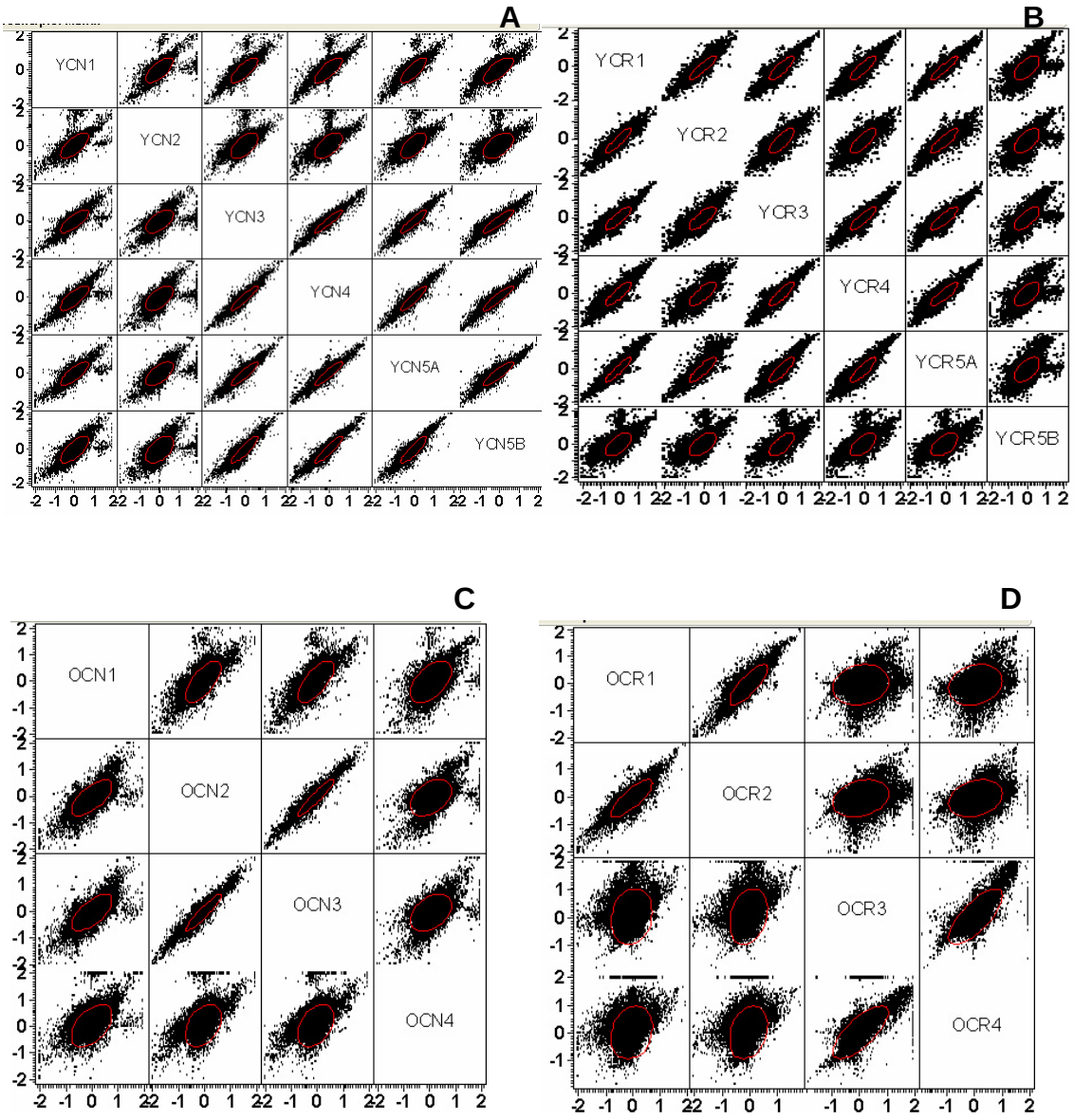


Figure 5.1 Replicate Correlation Analysis. Biological replicates for microarray analysis were determined based on shape and correlation coefficients for scatter plots within each group. YCON, YCR, OCON and OCR are shown above.

Table 5.4 Gene expression change comparisons. Comparisons between YCON versus YCR, YCON versus OCON, YCR versus OCR, OCON versus OCR, YCON versus OCR, and YCR versus OCON. e.g. There are 404 genes in which YCON is up-regulated as compared to YCR. 251 of the 404 identified genes have known functions.

Gene Change Comparison	Total Gene Changes	Gene Changes with Known Function
YCON > YCR	404	251
YCON < YCR	147	87
YCON > OCON	377	203
YCON < OCON	221	118
YCR > OCR	185	110
YCR < OCR	627	377
OCON > OCR	226	137
OCON < OCR	607	382
YCON > OCR	246	147
YCON < OCR	507	332
YCR > OCON	308	158
YCR < OCON	319	174

Table 5.5 Gene Changes in YCON versus YCR. Selected genes and molecular function for these genes A) up-regulated in YCON as compared to YCR or B) down-regulated in YCON as compared to YCR.

A

Gene Name	Molecular Function
Homo sapiens mitochondrial ribosomal protein S31 (MRPS31), nuclear gene encoding mitochondrial protein, mRNA	DNA binding
Homo sapiens transcription termination factor, mitochondrial (MTERF), nuclear gene encoding mitochondrial protein, mRNA	double-stranded DNA binding
Homo sapiens Chediak-Higashi syndrome 1 (CHS1), mRNA	electron transporter activity
Homo sapiens isocitrate dehydrogenase 3 (NAD+) beta (IDH3B), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA	electron transporter activity
Homo sapiens hypothetical protein FLJ12519 (FLJ12519), mRNA	electron transporter activity
Homo sapiens TNF receptor-associated factor 3 (TRAF3), transcript variant 1, mRNA	electron transporter activity
Homo sapiens IMP2 inner mitochondrial membrane protease-like (S. cerevisiae), mRNA (cDNA clone MGC:14829 IMAGE:4279910), complete cds	serine-type peptidase activity

B

Gene Name	Molecular Function
Homo sapiens uncoupling protein 1 (mitochondrial, proton carrier) (UCP1), nuclear gene encoding mitochondrial protein, mRNA	Binding
Homo sapiens clone 24525 mRNA sequence	electron transporter activity
Homo sapiens cDNA FLJ31721 fis, clone NT2RI2006667	electron transporter activity

Table 5.6 Gene Changes in YCON versus OCON. Select list of gene names and molecular functions for genes A) up-regulated in YCON as compared to OCON or B) down-regulated in YCON as compared to OCON.

A

Gene Name	Molecular Function
Homo sapiens BCL2-related ovarian killer (BOK), mRNA	apoptosis activator activity
Homo sapiens interleukin 10 (IL10), mRNA	apoptosis inhibitor activity
Homo sapiens histone deacetylase 1 (HDAC1), mRNA	apoptosis inhibitor activity
Homo sapiens mitochondrial ribosomal protein L20 (MRPL20), nuclear gene encoding mitochondrial protein, mRNA	RNA binding

B

Gene Name	Molecular Function
Homo sapiens Bcl-2 related ovarian killer (BOK) mRNA, complete cds	apoptosis activator activity
Homo sapiens mRNA; cDNA DKFZp586A181 (from clone DKFZp586A181); partial cds	apoptosis regulator activity
Homo sapiens DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase) (DFFB), mRNA	apoptosis regulator activity
Homo sapiens caspase 4, apoptosis-related cysteine protease (CASP4), transcript variant gamma, mRNA	apoptosis regulator activity
Homo sapiens putative homeodomain transcription factor 2, mRNA (cDNA clone IMAGE:5171332), complete cds	electron transporter activity
Homo sapiens acid fibroblast growth factor-like protein (GLIO703) mRNA, complete cds	growth factor activity
Homo sapiens urotensin 2 (UTS2), transcript variant 1, mRNA	hormone activity

Table 5.7 Gene Changes in YCR versus OCR. Select list of gene names and molecular functions for genes A) up-regulated in YCR as compared to OCR or B) down-regulated in YCR as compared to OCR.

A

Gene Name	Molecular Function
Homo sapiens baculoviral IAP repeat-containing 7 (livin) (BIRC7), transcript variant 2, mRNA	apoptosis inhibitor activity
Homo sapiens clone pp7122 unknown mRNA	ATP-binding cassette (ABC) transporter activity
Homo sapiens uncoupling protein 3 (mitochondrial, proton carrier) (UCP3), nuclear gene encoding mitochondrial protein, transcript variant long, mRNA	Binding
Homo sapiens solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24, mRNA (cDNA clone IMAGE:5753455), partial cds	calcium ion binding
Homo sapiens mRNA for FLJ00039 protein	electron transporter activity
Homo sapiens chromosome 13 open reading frame 18, mRNA (cDNA clone MGC:40256 IMAGE:5212065), complete cds	electron transporter activity
Homo sapiens mRNA; cDNA DKFZp686D02125 (from clone DKFZp686D02125)	electron transporter activity

B

Gene Name	Molecular Function
Homo sapiens Fas apoptotic inhibitory molecule (FAIM), mRNA	apoptosis inhibitor activity
Homo sapiens myeloid cell leukemia sequence 1 (BCL2-related), transcript variant 1, mRNA (cDNA clone MGC:1839 IMAGE:3138465), complete cds	apoptosis regulator activity
Homo sapiens myeloid cell leukemia sequence 1 (BCL2-related) (MCL1), transcript variant 1, mRNA	apoptosis regulator activity
Homo sapiens solute carrier family 25, member 27 (SLC25A27), nuclear gene encoding mitochondrial protein, mRNA	binding
Homo sapiens mRNA for KIAA1753 protein, partial cds	electron transporter activity
Homo sapiens hypothetical protein FLJ90430, mRNA (cDNA clone MGC:26916 IMAGE:4837196), complete cds	electron transporter activity
Homo sapiens hypothetical protein FLJ90430, mRNA (cDNA clone MGC:26916 IMAGE:4837196), complete cds	electron transporter activity
Homo sapiens KIAA0170 mRNA, partial cds	electron transporter activity
Homo sapiens TNF receptor-associated factor 3 (TRAF3), transcript variant 1, mRNA	electron transporter activity
Homo sapiens vascular endothelial growth factor B (VEGFB), mRNA	growth factor activity
Homo sapiens vascular endothelial growth factor B (VEGFB), mRNA	growth factor activity
Homo sapiens IMP2 inner mitochondrial membrane protease-like (S. cerevisiae), mRNA (cDNA clone MGC:14829 IMAGE:4279910), complete cds	serine-type peptidase activity
Homo sapiens estrogen-related receptor alpha (ESRRA), mRNA	steroid binding
Homo sapiens forkhead box P1 (FOXP1), mRNA	transcription factor activity

Table 5.8 Gene Changes in OCON versus OCR. Select list of gene names and molecular functions for genes A) up-regulated in OCON as compared to OCR or B) down-regulated in OCON as compared to OCR.

A

Gene Name	Molecular Function
Homo sapiens baculoviral IAP repeat-containing 7 (livin) (BIRC7), transcript variant 2, mRNA	apoptosis inhibitor activity
Homo sapiens caspase 4, apoptosis-related cysteine protease (CASP4), transcript variant gamma, mRNA	apoptosis regulator activity
Homo sapiens CARD only protein (COP), mRNA	apoptosis regulator activity
Homo sapiens uncoupling protein 3 (mitochondrial, proton carrier) (UCP3), nuclear gene encoding mitochondrial protein, transcript variant long, mRNA	binding
Homo sapiens caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase) (CASP1), transcript variant alpha, mRNA	caspase activator activity
Homo sapiens translocase of outer mitochondrial membrane 7 homolog (yeast) (TOMM7), mRNA	protein transporter activity

B

Gene Name	Molecular Function
Homo sapiens interleukin 18 (interferon-gamma-inducing factor) (IL18), mRNA	apoptosis activator activity
Homo sapiens Fas apoptotic inhibitory molecule (FAIM), mRNA	apoptosis inhibitor activity
Homo sapiens caspase 2, apoptosis-related cysteine protease (neural precursor cell expressed, developmentally down-regulated 2) (CASP2), transcript variant 1, mRNA	apoptosis inhibitor activity
Homo sapiens myeloid cell leukemia sequence 1 (BCL2-related), transcript variant 1, mRNA (cDNA clone MGC:1839 IMAGE:3138465), complete cds	apoptosis regulator activity
Homo sapiens phosphoprotein enriched in astrocytes 15 (PEA15), mRNA	apoptosis regulator activity
Homo sapiens myeloid cell leukemia sequence 1 (BCL2-related) (MCL1), transcript variant 1, mRNA	apoptosis regulator activity
Homo sapiens solute carrier family 25, member 27 (SLC25A27), nuclear gene encoding mitochondrial protein, mRNA	binding
Homo sapiens solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23 (SLC25A23), mRNA	calcium ion binding
Homo sapiens mRNA for KIAA1753 protein, partial cds	electron transporter activity
Homo sapiens clone 24525 mRNA sequence	electron transporter activity
Homo sapiens DKFZp434A0131 protein, mRNA (cDNA clone MGC:1130 IMAGE:3160366), complete cds	electron transporter activity
Homo sapiens hypothetical protein FLJ90430, mRNA (cDNA clone MGC:26916 IMAGE:4837196), complete cds	electron transporter activity
Homo sapiens hypothetical protein FLJ90430, mRNA (cDNA clone MGC:26916 IMAGE:4837196), complete cds	electron transporter activity
Homo sapiens KIAA0170 mRNA, partial cds	electron transporter activity
Homo sapiens nuclear prelamin A recognition factor (NARF), transcript variant 1, mRNA	electron transporter activity
Homo sapiens estrogen-related receptor alpha (ESRRA), mRNA	steroid binding
Homo sapiens forkhead box P1 (FOXP1), mRNA	transcription factor activity

Table 5.9 Gene changes in YCON versus OCR. Select list of gene names and molecular functions for genes A) up-regulated in YCON as compared to OCR or B) down-regulated in YCON as compared to OCR.

A

Gene Name	Molecular Function
Homo sapiens uncoupling protein 3 (mitochondrial, proton carrier) (UCP3), nuclear gene encoding mitochondrial protein, transcript variant long, mRNA	binding
Homo sapiens solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24, mRNA (cDNA clone IMAGE:5753455), partial cds	calcium ion binding
Homo sapiens caspase 1, apoptosis-related cysteine protease (interleukin 1, beta, convertase) (CASP1), transcript variant alpha, mRNA	caspase activator activity
Homo sapiens mRNA for FLJ00039 protein	electron transporter activity
Homo sapiens mRNA; cDNA DKFZp686E08116 (from clone DKFZp686E08116)	electron transporter activity
Homo sapiens chromosome 13 open reading frame 18, mRNA (cDNA clone MGC:40256 IMAGE:5212065), complete cds	electron transporter activity

B

Gene Name	Molecular Function
Homo sapiens Fas apoptotic inhibitory molecule (FAIM), mRNA	apoptosis inhibitor activity
Homo sapiens caspase 2, apoptosis-related cysteine protease (neural precursor cell expressed, developmentally down-regulated 2) (CASP2), transcript variant 1, mRNA	apoptosis inhibitor activity
Homo sapiens myeloid cell leukemia sequence 1 (BCL2-related), transcript variant 1, mRNA (cDNA clone MGC:1839 IMAGE:3138465), complete cds	apoptosis regulator activity
Homo sapiens phosphoprotein enriched in astrocytes 15 (PEA15), mRNA	apoptosis regulator activity
Homo sapiens DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase) (DFFB), mRNA	apoptosis regulator activity
Homo sapiens myeloid cell leukemia sequence 1 (BCL2-related) (MCL1), transcript variant 1, mRNA	apoptosis regulator activity
Homo sapiens BCL2 binding component 3 (BBC3), mRNA	catalytic activity
Homo sapiens 8-oxoguanine DNA glycosylase (OGG1), nuclear gene encoding mitochondrial protein, transcript variant 1b, mRNA	damaged DNA binding
Homo sapiens mRNA for KIAA1753 protein, partial cds	electron transporter activity
Homo sapiens clone 24525 mRNA sequence	electron transporter activity
Homo sapiens hypothetical protein FLJ90430, mRNA (cDNA clone MGC:26916 IMAGE:4837196), complete cds	electron transporter activity
Homo sapiens hypothetical protein FLJ90430, mRNA (cDNA clone MGC:26916 IMAGE:4837196), complete cds	electron transporter activity
Homo sapiens KIAA0170 mRNA, partial cds	electron transporter activity
Homo sapiens vascular endothelial growth factor B (VEGFB), mRNA	growth factor activity
Homo sapiens estrogen-related receptor alpha (ESRRA), mRNA	steroid binding
Homo sapiens forkhead box O1A (rhabdomyosarcoma) (FOXO1A), mRNA	transcription factor activity
Homo sapiens forkhead box P1 (FOXP1), mRNA	transcription factor activity

Table 5.10 Gene Changes in YCR versus OCON. Select list of gene names and molecular functions for genes A) up-regulated in YCR as compared to OCON or B) down-regulated in YCR as compared to OCON

.

A

Gene Name	Molecular Function
Homo sapiens solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 24, mRNA (cDNA clone IMAGE:5753455), partial cds	calcium ion binding
Homo sapiens clone 24525 mRNA sequence	electron transporter activity
Homo sapiens mRNA for FLJ00039 protein	electron transporter activity
Homo sapiens nuclear prelamin A recognition factor (NARF), transcript variant 1, mRNA	electron transporter activity
Homo sapiens forkhead box A1 (FOXA1), mRNA	transcription factor activity

B

Gene Name	Molecular Function
Homo sapiens DNA fragmentation factor, 40kDa, beta polypeptide (caspase-activated DNase) (DFFB), mRNA	apoptosis regulator activity
Homo sapiens putative homeodomain transcription factor 2, mRNA (cDNA clone IMAGE:5171332), complete cds	electron transporter activity
Homo sapiens hypothetical protein FLJ12519 (FLJ12519), mRNA	electron transporter activity
Homo sapiens TNF receptor-associated factor 3 (TRAF3), transcript variant 1, mRNA	electron transporter activity
Homo sapiens apoptosis-related protein PNAS-1 (FLJ39616), mRNA	myosin ATPase activity
Homo sapiens mitochondrial translational initiation factor 3 (MTIF3), mRNA	translation initiation factor activity

Table 5.11 Summary table of Gene Changes. List of specific genes highlighted as possible candidates for RT-PCR.

Gene Name	Molecular Function	Comparison
Homo sapiens caspase 4, apoptosis-related cysteine protease (CASP4), transcript variant gamma, mRNA	apoptosis regulator activity	YCON < OCON OCON > OCR
Homo sapiens Fas apoptotic inhibitory molecule (FAIM), mRNA	apoptosis inhibitor activity	YCR < OCR YCON < OCR OCON < OCR
Homo sapiens uncoupling protein 3 (mitochondrial, proton carrier) (UCP3), nuclear gene encoding mitochondrial protein, transcript variant long, mRNA	binding	YCON > OCR YCR > OCR OCON > OCR
Homo sapiens estrogen-related receptor alpha (ESRRA), mRNA	steroid binding	YCON < OCR YCR < OCR OCON < OCR
Homo sapiens forkhead box P1 (FOXP1), mRNA	transcription factor activity	YCR < OCR OCON < OCR
Homo sapiens forkhead box O1A (rhabdomyosarcoma) (FOXO1A), mRNA	transcription factor activity	YCON < OCR YCR > OCON

CHAPTER 6

GENERAL DISCUSSION

The objectives of this project were to: 1) analyze general hormonal changes that occur with aging and peri-menopause and 2) evaluate the effects (whether beneficial or detrimental) of moderate (30%) CR on ovarian function and the age-related decline in rhesus monkeys (*Macaca mulatta*). The results of our research are exceptionally promising and have given us insight into the use of the rhesus monkey model in menopause research, as well significant information on the normative aging processes of this animal. Furthermore, our findings of improved ovarian response and embryo development after COS in monkeys maintained on short-term, moderate CR provide valuable data on the effects of CR on normal reproductive function and aging.

The field of menopause research is still relatively new and several fundamental questions about menopause still remain to be answered. For example, what is the first sign of reproductive decline and is it possible to somehow retard or delay the onset of peri-menopause? Current models for studying reproductive aging include aged mouse and rat models, as well as the use of ovariectomized animals. Use of the aforementioned models has disadvantages, not the least of which is the absence of a “true” menopause. The trigger(s) for the onset of reproductive decline in women likely involves multiple systems and hormones. The discovery of elevated levels of FSH and reduced levels of INHB in women prior to menstrual cycle irregularities provides some

insight into the changes that occur with declining fertility. Unfortunately, much of our data on reduced fertility and hormone changes are derived from human fertility clinics addressing reproductive malfunction accompanying ovarian pathology or advanced age.

There are few studies reporting the normal progression of ovarian decline in women. Thus, validation of the rhesus monkey as an appropriate model for menopause is extremely beneficial both to the human and nonhuman primate research communities. Insight into the normative aging processes involved in pre-, peri- and post-menopause will help us to understand the progression of this natural and inevitable life stage. Rhesus monkeys have been widely used as an animal model for human reproduction, due to its similarity in menstrual cycles and hormone profiles (Heape, 1900). Gilardi and colleagues' (Gilardi et al., 1997) paper characterizing urinary hormones during the peri-menopause brought into question, however, the utility of the rhesus monkey as a model for human menopause. Their research suggested that one of the hallmark events of human menopause, namely the monotropic rise in follicle-stimulating hormone (FSH; (Klein et al., 1996a; Klein et al., 1996b; Klein et al., 1998; Prior, 2005), occurred in rhesus monkeys only after menstrual cycle irregularity was observed. Our research, however, has proven that is not the case. In fact, not only did we detect changes in FSH prior to menstrual cycle irregularity, but changes in inhibin B (INHB) were also detectable. These data are the first to measure hormone profiles in older rhesus monkeys in a semi-longitudinal fashion and confirm parallel processes of ovarian function with entry into the peri-menopause.

Similar to humans, rhesus monkeys can also have varying responses to controlled ovarian stimulation (COS). Menstrual cycle history alone is not sufficient to make predictions on the likelihood of a successful stimulation by exogenous gonadotropins. Even among the regularly cycling females, we observed mixed responses to COS. A single COS cycle in rhesus monkeys is approximately $1000 for reagents alone. Furthermore, the cost of a failed COS is not only monetary; in dealing with aging females and ovarian senescence, timing is essential. Therefore, the utility of developing an effective and accurate ovarian reserve test (ORT) in monkeys is obvious and necessary.

The efficacy of several commonly used ORTs in human clinics proved to be very successful when applied in rhesus monkeys. Prior to this report, there has been very little research evaluating ORTs in nonhuman primates (Marut and Hodgen, 1982). Surprisingly, our results have shown that FSH is not the most accurate hormone measure of response to COS. In fact, INHB was consistently more accurate, regardless of the ORT. Similar to our first experiment, we found the CON and CR (short-term, ONPRC) responses to the ORTs to be similar.

Using these data on normative aging processes in rhesus monkeys we were further able to evaluate the effects of CR on normal reproductive function. Calorie restriction (CR) has long been heralded as the only proven nutritional intervention for life extension. The benefits of CR were first described in 1935 (McCay et al., 1935) and have since been shown to be effective in a number of species, including roundworms, fruit flies, mice and rats (Ingram et al., 2004; Mattison et al., 2003; Merry, 2004; Roth et al., 1999; Smith et al., 2004). Current

investigations are underway, on whether CR will extend lifespan and/or improve health in rhesus monkeys.

Given the close relationship between nutrition, body fat and reproduction (Bray, 1997; Kiess et al., 1999; Messinis and Milingos, 1999; Misra et al., 2004), it was important to evaluate whether 30% CR would disrupt normal ovarian function. Previous data has demonstrated that moderate extends reproductive lifespan in female rats (Holehan and Merry, 1985b; Merry and Holehan, 1985). In mice, however, extension of reproductive lifespan was only observed after re-feeding animals; normal cycling was disrupted by the CR diet (Nelson et al., 1985). Our data demonstrated that moderate CR (30%) did not interfere with normal menstrual cycling in our rhesus monkeys. We did not, however, find conclusive evidence to indicate that moderate CR had any effect (either beneficial or detrimental) on the onset of peri-menopause in the long-term (NIA) monkeys. Menses records taken on the short-term (ONPRC) monkeys suggest that initiation of CR in old monkeys may not have detrimental effects within the first year or two on the dietary treatment; however, by year 3, there are signs of advancement into peri-menopause.

Embryo development following COS in our old CON and CR monkeys (short-term, ONPRC) provided surprising responses. Oocyte quality is known to diminish with age (Coticchio et al., 2004; Esfandiari et al., 2005; Navot et al., 1991; Navot et al., 1994), although the reasons for this are not fully understood. There are a variety of elements that may play a role in the reduction of oocyte quality, including external factors such as granulosa cells, follicular fluid, ovarian

stimulus from the pituitary; as well as internal factors such as mitochondria, spindle assembly and cytoplasm.

Our data showed similar responses between the CON and CR the number of oocytes collected, oocyte maturity and fertilization. Response to the COS was divided into “good,” “poor,” or “non” responders. Surprisingly, the age of poor responders was higher in the CON as compared to the CR group, suggesting an improved responsiveness to exogenous gonadotropins. Furthermore, we found that embryo development was improved in oocytes collected from CR monkeys as compared to CON. In fact, none of the CON embryos developed past the blastocyst stage, leading us to conclude that short-term exposure to moderate CR in old female rhesus monkeys has beneficial effects on ovarian function.

Finally, our preliminary findings using microarray analyses to examine gene expression in luteinizing granulosa cells suggest a number of exciting gene changes that may help to explain the mechanism of action of CR on ovarian function. These include apoptotic-, mitochondrial-, and steroid binding- related gene functions. These data fit in well given current hypotheses regarding possible mechanisms of action of CR: protection from damage by free radical production or gene silencing and/or activation.

There are a number of other possible directions we can pursue based on the results of this work. In particular, the long-term (NIA) CR group of monkeys is part of a longevity study; and as such will be monitored through to mortality. Unfortunately, we will be unable to take further hormone measurements in the short-term (ONPRC) CR group of monkeys.

Based on our results, we recommend a protocol for the long-term (NIA) group of monkeys that would continue over the course of the animals' lifetimes that would require a minimum of effort for the greatest return. Daily monitoring of onset of menses and continued record keeping of menstrual cycles provide a relatively simple and non-invasive measure of reproductive function. Secondly, our data suggest that a single blood collection on menstrual cycle day 3 (onset of menses = day 1), measured for FSH and INHB can confer a great deal of information regarding the female's reproductive status. A second blood collection taken on day 21 for progesterone (P4) would confirm ovulation, especially necessary with aging females. We would suggest collecting these samples two to three times a year during the normal breeding season (September through June). Additionally, our modified clomiphene citrate challenge test (CCCT; day 3 blood collection for INHB and E2; 50 mg CC daily, on days 5-9; day 10 blood collection for estradiol) may be implemented subsequent to one of the scheduled blood collections at least once per year. Combined, these data would provide a unique longitudinal record of the ovarian function in aging and long-term (NIA) CR monkeys.

Additionally, given the intriguing embryo development data collected from the short-term (ONPRC) CR monkeys, it would be especially enlightening to perform a similar experiment in our long-term (NIA) CR colony. By first performing the modified CCCT, we would be able to selectively choose those animals most likely to respond to COS and yield eggs via follicle aspiration. Since the inception of this study, a number of commercially available kits have

become available permitting the extraction of high quality RNA from very small samples. Thus, we recommend not only collecting luteinizing granulosa cells (LGCs) for microarray analysis, but also oocytes (half reserved for microarray and half for the embryo development studies). This will shed light on two questions: 1) whether long-term CR has similar effects on embryo development and 2) whether differential gene expression is more evident in the LGCs, or the oocyte itself.

Having the opportunity to revisit this experiment in retrospect, there are a few methodological points that should be considered if a repeat is possible. A medical issue that was not mentioned in this thesis is the issue of incidence of endometriosis. We did not anticipate the loss of females from this disease and as such, would recommend addition of animals at the outset of the study, accounting for a percentage loss from this pernicious illness. Further studies investigating the effects of CR on endometriosis must also be considered. Secondly, without menses records taken from the long-term (NIA) females at the outset of the study, it is impossible to know whether some, or the entire "young" group of monkeys were pre- or post-pubertal when CR was initiated. It is unknown whether reproductive status at CR onset would affect our results; however, it would at least provide a homogenous population of females. Furthermore, in addressing the issue of homogenous groups, it would be useful in the future to perform ORTs on any "old" group of monkeys. This would allow investigators to separate out pre- or peri-menopausal females as it relates to their studies. Finally, although rhesus monkeys have been used widely for

studies in reproduction, it would be wise to consider the use of cynomologous monkeys in the future. Rhesus monkeys are seasonal breeders, even when housed indoors, and reproductive data cannot be collected during the summer months. Alternatively, cynomologous monkeys will continue to cycle year-round and may be a more flexible model with similar advantages.

There are still a number of unanswered questions left for investigators in this field to uncover. To fully understand how we can delay menopause, we must first answer the question: how does menopause occur? Furthermore, if in fact CR can delay the onset of peri-menopause, it would be of interest whether CR simply delays the final menses or if it changes the curve of reproductive decline as well. That is, would CR also extend the period of time before the very first sign of declining fertility or simply change the slope of the decline?

A highly charged topic in menopause research, is the question of whether the brain or the ovary ages first? Data suggesting that INHB levels begin to decline prior to elevations in FSH suggest that perhaps the first change occurs at the level of the ovary. Extraction and isolation of rhesus monkey FSH and INHB would permit a simple experiment: hormone replacement of one or both of these hormones at the point when we are first able to detect differences. A hormone replacement regimen for women with declining fertility would be extremely useful.

With the development of new methods of transplanting ovarian tissue (especially for use with cancer patients), it would be intriguing to test transplantation of ovarian tissue from control animals into CR and vice versa.

This may provide some useful information regarding feedback systems between the ovary and the hypothalamus and pituitary, especially in animals maintained on CR. One of the questions that comes to mind in reviewing our embryo development data, is when does CR exert its effect on potentially improving oocyte quality? That is, is CR affecting oocyte development from recruitment from the primordial follicle or at later stages: primary, secondary or tertiary stages? If we could transplant a larger quantity of ovarian tissue from a CON into a CR monkey and subsequently administered a successful COS, would you see the same embryo development effects found in oocytes collected directly from the ovary? And would you cancel those effects by transplanting tissue from a CR into a CON monkey?

We have discussed the possibility that CR effects on mitochondrial function could have contributed to the benefits observed in embryo development. Certainly microarray analysis of oocytes collected from CON and CR monkeys would yield valuable data and changes in specific genes could be pursued, including but not limited to sirtuins, forkhead transcription factors, bcl-2 family of genes and of course metabolism related genes. Additionally, since mitochondria are so rich in the oocyte cytoplasm, cytoplasmic transfer experiments could be performed. Essentially, this involves the transfer of cytoplasmic material from one egg into another. In humans, this procedure has mixed reviews, in particular ethical concerns regarding the transfer of mitochondrial DNA from a third party into the egg. In monkeys, however, it may offer some insight into the contribution

of cytoplasmic mitochondria collected from CR oocytes and the effect it may have on CON oocytes.

Historically, it has been believed that female vertebrates have the complete population of ovarian follicles at or around the time of birth. Furthermore, the near depletion of the follicular reserve by menopause has been explained by a bi-exponential loss of follicles that is greatly accelerated at approximately 40 years of age (vom Saal et al., 1994). Johnson, and colleagues (Johnson et al., 2004) have brought that doctrine into question with recent data suggesting the possible de novo synthesis of oocytes and follicles in adult mice. There is also controversy regarding the accurate measurement of ovarian follicles and whether the bi-exponential loss of follicles is a true measure of follicular depletion. Thus, two questions come to mind, including what exactly are the morphometric follicular changes occurring in the ovary at peri-menopause and does CR affect those changes? Additionally, if in fact Johnson and colleagues are correct in their report that de novo oocyte synthesis is possible, is CR acting by re-populating the follicular reserves, rather than by maintaining them? Identification of a gene that can “jump-start” oocyte and follicular production would be worth its weight in gold for women who have found themselves in infertility clinics at 35 years age.

It would also be wonderful to systematically ovariectomize rhesus monkeys at specific reproductive life stages: birth, pre- and post-pubertal, pre-, peri- and post-menopausal. We could identify the appropriate monkeys at each menopausal stage by using menses records, day 3 INHB and/or the modified

CCCT. In this way, we could capture snapshot records of ovarian morphology and follicular populations (including data on primordial, primary, secondary follicles, etc). Performing this same experiment using both CON and CR would help to uncover how CR is affecting ovarian health and aging. Perhaps it would be even more useful to perform this experiment first on mice and rats; this may assist in elucidating the differences found between these two species with regard to continued or disrupted cycling with implementation of CR.

We do not yet know whether moderate CR will delay menopause in the long-term (NIA) monkeys. Rodent data are mixed with regard to ovarian response to CR. Perhaps rhesus monkeys will be more “rat”-like and demonstrate delayed reproductive aging. Conversely, rhesus monkeys may be more “mouse”-like and require menstrual cycle disruption to elicit the same response. It would be interesting to initiate CR levels high enough to disrupt normal menstrual cycling. Long-term acyclicity, followed by re-feeding may show more dramatic results in rhesus monkeys than long-term maintenance on moderate CR. If results indicated that long-term acyclicity did improve or maintain reproductive function with re-feeding, it may be more practical to consider the use of long-term gonadotropin releasing hormone (GnRH) antagonists. Limited data have been published suggesting the efficacy of ovarian suppression in ovo-protection during chemotherapy. Perhaps CR effects on extending reproductive lifespan in mice works via a similar mechanism.

In keeping with our findings that suggest improved embryo quality in monkeys maintained on CR, it would be extremely useful to also perform

fluorescent in situ hybridization (FISH) identification on chromosomes in the oocytes collected following COS. Aneuploidy and trisomy increases with age of conception in women; thus analysis of oocytes collected from CON and CR monkeys would further elucidate questions about egg quality and possible chromosomal abnormalities in these monkeys.

The prospect of delaying menopause has wider reaching impact than simply extending reproductive lifespan. A number of menopause-related diseases are major health issues for women over 50, including cardiovascular, cognitive, skeletal issues (Gosden, 1985; Nappi et al., 1999; Prior, 1998; Sherwin, 2003). Thus, further understanding of CR and its effect on ovarian function is timely and relevant. Although implementing moderate CR in women would be a difficult lifestyle change, in the short-term it may be feasible. Furthermore, the development of CR mimetics is currently underway and may be a useful means of acquiring the benefits of CR without the dietary regimen. We hope that this research may serve as a foundation and launching point for future work in this area.

REFERENCES

Abma, J. C., Chandra, A., Mosher, W. D., Peterson, L. S., and Piccinino, L. J. (1997). Fertility, family planning, and women's health: new data from the 1995 National Survey of Family Growth. Vital Health Stat 23, 1-114.

Agarwal, A., Gupta, S., and Sharma, R. (2005). Oxidative stress and its implications in female infertility - a clinician's perspective. Reprod Biomed Online *11*, 641-650.

Akande, V. A., Keay, S. D., Hunt, L. P., Mathur, R. S., Jenkins, J. M., and Cahill, D. J. (2004). The practical implications of a raised serum FSH and age on the risk of IVF treatment cancellation due to a poor ovarian response. J Assist Reprod Genet *21*, 257-262.

Al-Qahtani, A., Muttukrishna, S., Appasamy, M., Johns, J., Cranfield, M., Visser, J. A., Themmen, A. P., and Groome, N. P. (2005). Development of a sensitive enzyme immunoassay for anti-Mullerian hormone and the evaluation of potential clinical applications in males and females. Clin Endocrinol (Oxf) *63*, 267-273.

Alexander, R. D. (1974). The evolution of social behavior. Annual Review of Ecology and Systematics *5*, 325-383.

Anzalone, C. R., Hong, L. S., Lu, J. K., and LaPolt, P. S. (2001). Influences of age and ovarian follicular reserve on estrous cycle patterns, ovulation, and hormone secretion in the Long-Evans rat. Biol Reprod *64*, 1056-1062.

Badaru, A., Wilson, D. M., Bachrach, L. K., Fechner, P., Gandrud, L. M., Durham, E., Wintergerst, K., Chi, C., Klein, K. O., and Kirk Neely, E. (2006).

Sequential Comparisons of 1-Month and 3-Month Depot Leuprolide Regimens in Central Precocious Puberty. J Clin Endocrinol Metab.

Balmaceda, J. P., Bernardini, L., Ciuffardi, I., Felix, C., Ord, T., Sueldo, C. E., and Asch, R. H. (1994). Oocyte donation in humans: a model to study the effect of age on embryo implantation rate. Hum Reprod *9*, 2160-2163.

Banham, A. H., Beasley, N., Campo, E., Fernandez, P. L., Fidler, C., Gatter, K., Jones, M., Mason, D. Y., Prime, J. E., Trougouboff, P.*, et al.* (2001). The FOXP1 winged helix transcription factor is a novel candidate tumor suppressor gene on chromosome 3p. Cancer Res *61*, 8820-8829.

Barroso, G., Barrionuevo, M., Rao, P., Graham, L., Danforth, D., Huey, S., Abuhamad, A., and Oehninger, S. (1999). Vascular endothelial growth factor, nitric oxide, and leptin follicular fluid levels correlate negatively with embryo quality in IVF patients. Fertil Steril *72*, 1024-1026.

Bechmann, I., Diano, S., Warden, C. H., Bartfai, T., Nitsch, R., and Horvath, T. L. (2002). Brain mitochondrial uncoupling protein 2 (UCP2): a protective stress signal in neuronal injury. Biochem Pharmacol *64*, 363-367.

Bellino, F. L., and Wise, P. M. (2003). Nonhuman primate models of menopause workshop. Biol Reprod *68*, 10-18.

Billig, H., Furuta, I., and Hsueh, A. J. (1994). Gonadotropin-releasing hormone directly induces apoptotic cell death in the rat ovary: biochemical and in situ detection of deoxyribonucleic acid fragmentation in granulosa cells. Endocrinology *134*, 245-252.

Black, A., Allison, D. B., Shapses, S. A., Tilmont, E. M., Handy, A. M., Ingram, D. K., Roth, G. S., and Lane, M. A. (2001). Calorie restriction and skeletal mass in rhesus monkeys (Macaca mulatta): evidence for an effect mediated through changes in body size. J Gerontol A Biol Sci Med Sci *56*, B98-107.

Blurton Jones, N. G., Hawkes, K., and O'Connell, J. F. (2002). Antiquity of postreproductive life: are there modern impacts on hunter-gatherer postreproductive life spans? Am J Hum Biol *14*, 184-205.

Bois, P. R., and Grosveld, G. C. (2003). FKHR (FOXO1a) is required for myotube fusion of primary mouse myoblasts. Embo J *22*, 1147-1157.

Bois, P. R., Izeradjene, K., Houghton, P. J., Cleveland, J. L., Houghton, J. A., and Grosveld, G. C. (2005). FOXO1a acts as a selective tumor suppressor in alveolar rhabdomyosarcoma. J Cell Biol *170*, 903-912.

Borman, S. M., VanDePol, B. J., Kao, S., Thompson, K. E., Sipes, I. G., and Hoyer, P. B. (1999). A single dose of the ovotoxicant 4-vinylcyclohexene diepoxide is protective in rat primary ovarian follicles. Toxicol Appl Pharmacol *158*, 244-252.

Bray, G. A. (1997). Obesity and reproduction. Hum Reprod *12 Suppl 1*, 26-32.

Brenkman, A. B., and Burgering, B. M. (2003). FoxO3a eggs on fertility and aging. Trends Mol Med *9*, 464-467.

Broekmans, F. J., Faddy, M. J., Scheffer, G., and te Velde, E. R. (2004). Antral follicle counts are related to age at natural fertility loss and age at menopause. Menopause *11*, 607-614.

Broekmans, F. J., and Klinkert, E. R. (2004). Female age in ART: when to stop? Gynecol Obstet Invest *58*, 225-234.

Bukman, A., and Heineman, M. J. (2001). Ovarian reserve testing and the use of prognostic models in patients with subfertility. Hum Reprod Update *7*, 581-590.

Burgering, B. M., and Medema, R. H. (2003). Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty. J Leukoc Biol *73*, 689-701.

Camp, T. A., Rahal, J. O., and Mayo, K. E. (1991). Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNAs in the rat ovary. Mol Endocrinol *5*, 1405-1417.

Castrillon, D. H., Miao, L., Kollipara, R., Horner, J. W., and DePinho, R. A. (2003). Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science *301*, 215-218.

Chin, K. V., Seifer, D. B., Feng, B., Lin, Y., and Shih, W. C. (2002). DNA microarray analysis of the expression profiles of luteinized granulosa cells as a function of ovarian reserve. Fertil Steril *77*, 1214-1218.

Clavero, A., Castilla, J. A., Nunez, A. I., Garcia-Pena, M. L., Maldonado, V., Fontes, J., Mendoza, N., and Martinez, L. (2003). Apoptosis in human granulosa cells after induction of ovulation in women participating in an intracytoplasmic sperm injection program. Eur J Obstet Gynecol Reprod Biol *110*, 181-185.

Collins, D. C., Graham, C. E., and Preedy, J. R. (1975). Identification and measurement of urinary estrone, estradiol-17 beta, estriol, pregnanediol and androsterone during the menstrual cycle of the orangutan. Endocrinology *96*, 93-101.

Colman, R. J., Kemnitz, J. W., Lane, M. A., Abbott, D. H., and Binkley, N. (1999). Skeletal effects of aging and menopausal status in female rhesus macaques. J Clin Endocrinol Metab *84*, 4144-4148.

Conti, B., Sugama, S., Lucero, J., Winsky-Sommerer, R., Wirz, S. A., Maher, P., Andrews, Z., Barr, A. M., Morale, M. C., Paneda, C.*, et al.* (2005). Uncoupling protein 2 protects dopaminergic neurons from acute 1,2,3,6-methyl-phenyl-tetrahydropyridine toxicity. J Neurochem *93*, 493-501.

Coticchio, G., Sereni, E., Serrao, L., Mazzone, S., Iadarola, I., and Borini, A. (2004). What criteria for the definition of oocyte quality? Ann N Y Acad Sci *1034*, 132-144.

Cunningham, M. A., Zhu, Q., and Hammond, J. M. (2004). FoxO1a can alter cell cycle progression by regulating the nuclear localization of p27kip in granulosa cells. Mol Endocrinol *18*, 1756-1767.

Dahl, K. D., Czekala, N. M., Lim, P., and Hsueh, A. J. (1987). Monitoring the menstrual cycle of humans and lowland gorillas based on urinary profiles of bioactive follicle-stimulating hormone and steroid metabolites. J Clin Endocrinol Metab *64*, 486-493.

Danforth, D. R., Arbogast, L. K., Mroueh, J., Kim, M. H., Kennard, E. A., Seifer, D. B., and Friedman, C. I. (1998). Dimeric inhibin: a direct marker of ovarian aging. Fertil Steril *70*, 119-123.

Danilovich, N., Javeshghani, D., Xing, W., and Sairam, M. R. (2002). Endocrine alterations and signaling changes associated with declining ovarian function and advanced biological aging in follicle-stimulating hormone receptor haploinsufficient mice. Biol Reprod *67*, 370-378.

Danilovich, N., and Sairam, M. R. (2002). Haploinsufficiency of the follicle-stimulating hormone receptor accelerates oocyte loss inducing early reproductive senescence and biological aging in mice. Biol Reprod *67*, 361-369.

Danilovich, N., Sairam, M. R., and Maysinger, D. (2003). The menopausal mouse: a new neural paradigm of a distressing human condition. Neuroreport *14*, 1617-1622.

de Souza, C. J., Campbell, B. K., and Baird, D. T. (1998). Incipient ovarian failure associated with raised levels of follicle stimulating hormone and reduced levels of inhibin A in older sheep. Hum Reprod *13*, 3016-3022.

Dierich, A., Sairam, M. R., Monaco, L., Fimia, G. M., Gansmuller, A., LeMeur, M., and Sassone-Corsi, P. (1998). Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci U S A *95*, 13612-13617.

Doerr, J. K., Hooser, S. B., Smith, B. J., and Sipes, I. G. (1995). Ovarian toxicity of 4-vinylcyclohexene and related olefins in B6C3F1 mice: role of diepoxides. Chem Res Toxicol *8*, 963-969.

Domb, L. G., and Pagel, M. (2001). Sexual swellings advertise female quality in wild baboons. Nature *410*, 204-206.

Driancourt, M. A., Reynaud, K., Cortvrindt, R., and Smitz, J. (2000). Roles of KIT and KIT LIGAND in ovarian function. Rev Reprod *5*, 143-152.

Dzik, A., Lambert-Messerlian, G., Izzo, V. M., Soares, J. B., Pinotti, J. A., and Seifer, D. B. (2000). Inhibin B response to EFORT is associated with the outcome of oocyte retrieval in the subsequent in vitro fertilization cycle. Fertil Steril *74*, 1114-1117.

Esfandiari, N., Ryan, E. A., Gotlieb, L., and Casper, R. F. (2005). Successful pregnancy following transfer of embryos from oocytes with abnormal zona pellucida and cytoplasm morphology. Reprod Biomed Online *11*, 620-623.

Faddy, M. J., Gosden, R. G., Gougeon, A., Richardson, S. J., and Nelson, J. F. (1992). Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Hum Reprod *7*, 1342-1346.

Fanchin, R., de Ziegler, D., Olivennes, F., Taieb, J., Dzik, A., and Frydman, R. (1994). Exogenous follicle stimulating hormone ovarian reserve test (EFORT): a simple and reliable screening test for detecting 'poor responders' in in-vitro fertilization. Hum Reprod *9*, 1607-1611.

Felicio, L. S., Nelson, J. F., and Finch, C. E. (1984). Longitudinal studies of estrous cyclicity in aging C57BL/6J mice: II. Cessation of cyclicity and the duration of persistent vaginal cornification. Biol Reprod *31*, 446-453.

Fulka, J., Jr., Loi, P., Ledda, S., Moor, R. M., and Fulka, J. (2001). Nucleus transfer in mammals: how the oocyte cytoplasm modifies the transferred nucleus. Theriogenology *55*, 1373-1380.

Fulka, J., Jr., Mrazek, M., Fulka, H., and Loi, P. (2005). Mammalian oocyte therapies. Cloning Stem Cells *7*, 183-188.

Garcia-Saez, A. J., Coraiola, M., Dalla Serra, M., Mingarro, I., Menestrina, G., and Salgado, J. (2005). Peptides derived from apoptotic Bax and Bid reproduce the poration activity of the parent full-length proteins. Biophys J *88*, 3976-3990.

Gibbons, A. (1997). Anthropology: Why life after menopause? Science *276*, 535.

Gilardi, K. V., Shideler, S. E., Valverde, C. R., Roberts, J. A., and Lasley, B. L. (1997). Characterization of the onset of menopause in the rhesus macaque. Biol Reprod *57*, 335-340.

Gosden, R. G. (1985). The Biology of Menopause: The causes and consequences of ovarian aging (New York: Academic Press).

Gosden, R. G., Laing, S. C., Flurkey, K., and Finch, C. E. (1983). Graafian follicle growth and replacement in anovulatory ovaries of ageing C57BL/6J mice. J Reprod Fertil *69*, 453-462.

Gould, K. G., Flint, M., and Graham, C. E. (1981). Chimpanzee reproductive senescence: a possible model for evolution of the menopause. Maturitas *3*, 157-166.

Goya, L., Garcia-Segura, L. M., Ramos, S., Pascual-Leone, A. M., Argente, J., Martin, M. A., and Chowen, J. A. (2002). Interaction between malnutrition and ovarian hormones on the systemic IGF-I axis. Eur J Endocrinol *147*, 417-424.

Gresl, T. A., Colman, R. J., Roecker, E. B., Havighurst, T. C., Huang, Z., Allison, D. B., Bergman, R. N., and Kemnitz, J. W. (2001). Dietary restriction and glucose regulation in aging rhesus monkeys: a follow-up report at 8.5 yr. Am J Physiol Endocrinol Metab *281*, E757-765.

Groome, N. P., Illingworth, P. J., O'Brien, M., Cooke, I., Ganesan, T. S., Baird, D. T., and McNeilly, A. S. (1994). Detection of dimeric inhibin throughout the human menstrual cycle by two-site enzyme immunoassay. Clin Endocrinol (Oxf) *40*, 717-723.

Groome, N. P., Illingworth, P. J., O'Brien, M., Pai, R., Rodger, F. E., Mather, J. P., and McNeilly, A. S. (1996). Measurement of dimeric inhibin B throughout the human menstrual cycle. J Clin Endocrinol Metab *81*, 1401-1405.

Harber, V. J. (2000). Menstrual dysfunction in athletes: an energetic challenge. Exerc Sport Sci Rev *28*, 19-23.

Harber, V. J. (2004). Energy balance and reproductive function in active women. Can J Appl Physiol *29*, 48-58.

Hardy, K., Handyside, A. H., and Winston, R. M. (1989). The human blastocyst: cell number, death and allocation during late preimplantation development in vitro. Development *107*, 597-604.

Hawkes, K., O'Connell, J. F., Jones, N. G., Alvarez, H., and Charnov, E. L. (1998). Grandmothering, menopause, and the evolution of human life histories. Proc Natl Acad Sci U S A *95*, 1336-1339.

Heape, W. (1900). The sexual season of mammals. Q J Microsc Sci *44:1-70*.

Hess, D. L., Spies, H. G., and Hendrickx, A. G. (1981). Diurnal steroid patterns during gestation in the rhesus macaque: onset, daily variation, and the effects of dexamethasone treatment. Biol Reprod *24*, 609-616.

Hibbert, M. L., Stouffer, R. L., Wolf, D. P., and Zelinski-Wooten, M. B. (1996). Midcycle administration of a progesterone synthesis inhibitor prevents ovulation in primates. Proc Natl Acad Sci U S A *93*, 1897-1901.

Hillier, S. G. (2001). Gonadotropic control of ovarian follicular growth and development. Mol Cell Endocrinol *179*, 39-46.

Hitomi, J., Katayama, T., Eguchi, Y., Kudo, T., Taniguchi, M., Koyama, Y., Manabe, T., Yamagishi, S., Bando, Y., Imaizumi, K.*, et al.* (2004). Involvement of caspase-4 in endoplasmic reticulum stress-induced apoptosis and Abeta-induced cell death. J Cell Biol *165*, 347-356.

Hofmann, G. E., Danforth, D. R., and Seifer, D. B. (1998). Inhibin-B: the physiologic basis of the clomiphene citrate challenge test for ovarian reserve screening. Fertil Steril *69*, 474-477.

Holehan, A. M., and Merry, B. J. (1985a). The control of puberty in the dietary restricted female rat. Mech Ageing Dev *32*, 179-191.

Holehan, A. M., and Merry, B. J. (1985b). Lifetime breeding studies in fully fed and dietary restricted female CFY Sprague-Dawley rats. 1. Effect of age, housing conditions and diet on fecundity. Mech Ageing Dev *33*, 19-28.

Holehan, A. M., and Merry, B. J. (1985c). Modification of the oestrous cycle hormonal profile by dietary restriction. Mech Ageing Dev *32*, 63-76.

Hooser, S. B., Douds, D. P., DeMerell, D. G., Hoyer, P. B., and Sipes, I. G. (1994). Long-term ovarian and gonadotropin changes in mice exposed to 4-vinylcyclohexene. Reprod Toxicol *8*, 315-323.

Hosaka, T., Biggs, W. H., 3rd, Tieu, D., Boyer, A. D., Varki, N. M., Cavenee, W. K., and Arden, K. C. (2004). Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc Natl Acad Sci U S A *101*, 2975-2980.

Hoyer, P. B., Devine, P. J., Hu, X., Thompson, K. E., and Sipes, I. G. (2001). Ovarian toxicity of 4-vinylcyclohexene diepoxide: a mechanistic model. Toxicol Pathol *29*, 91-99.

Hsu, Y. T., Wolter, K. G., and Youle, R. J. (1997). Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. Proc Natl Acad Sci U S A *94*, 3668-3672.

Hu, X., Christian, P. J., Thompson, K. E., Sipes, I. G., and Hoyer, P. B. (2001). Apoptosis induced in rats by 4-vinylcyclohexene diepoxide is associated with activation of the caspase cascades. Biol Reprod *65*, 87-93.

Hutt, K. J., McLaughlin, E. A., and Holland, M. K. (2006). Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis. Mol Hum Reprod.

Ingram, D. K., Anson, R. M., de Cabo, R., Mamczarz, J., Zhu, M., Mattison, J., Lane, M. A., and Roth, G. S. (2004). Development of calorie restriction mimetics as a prolongevity strategy. Ann N Y Acad Sci *1019*, 412-423.

Ingram, D. K., Cutler, R. G., Weindruch, R., Renquist, D. M., Knapka, J. J., April, M., Belcher, C. T., Clark, M. A., Hatcherson, C. D., Marriott, B. M., and et al. (1990). Dietary restriction and aging: the initiation of a primate study. J Gerontol *45*, B148-163.

Jansen, E., Laven, J. S., Dommerholt, H. B., Polman, J., van Rijt, C., van den Hurk, C., Westland, J., Mosselman, S., and Fauser, B. C. (2004). Abnormal gene expression profiles in human ovaries from polycystic ovary syndrome patients. Mol Endocrinol *18*, 3050-3063.

Jansen, R. P., and Burton, G. J. (2004). Mitochondrial dysfunction in reproduction. Mitochondrion *4*, 577-600.

Johnson, A. L. (2000a). Granulosa cell apoptosis: conservation of cell signaling in an avian ovarian model system. Biol Signals Recept *9*, 96-101.

Johnson, A. L. (2000b). Reproduction in the female, In Sturkie's Avian Physiology, G. C. Whittow, ed. (San Diego: Academic Press), pp. 569-596.

Johnson, A. L. (2003). Intracellular mechanisms regulating cell survival in ovarian follicles. Anim Reprod Sci *78*, 185-201.

Johnson, J., Canning, J., Kaneko, T., Pru, J. K., Tilly, J. L., Packer, C., Tatar, M., Collins, A., Blurton Jones, N. G., Hawkes, K., and O'Connell, J. F. (2004). Germline stem cells and follicular renewal in the postnatal mammalian ovary

Reproductive cessation in female mammals

Antiquity of postreproductive life: are there modern impacts on hunter-gatherer postreproductive life spans? Nature *428*, 145-150.

Jonsson, H., and Peng, S. L. (2005). Forkhead transcription factors in immunology. Cell Mol Life Sci *62*, 397-409.

Jurisicova, A., Latham, K. E., Casper, R. F., and Varmuza, S. L. (1998). Expression and regulation of genes associated with cell death during murine preimplantation embryo development. Mol Reprod Dev *51*, 243-253.

Kahraman, S., Vicdan, K., Isik, A. Z., Ozgun, O. D., Alaybeyoglu, L., Polat, G., and Biberoglu, K. (1997). Clomiphene citrate challenge test in the assessment of ovarian reserve before controlled ovarian hyperstimulation for intracytoplasmic sperm injection. Eur J Obstet Gynecol Reprod Biol *73*, 177-182.

Kaipia, A., and Hsueh, A. J. (1997). Regulation of ovarian follicle atresia. Annu Rev Physiol *59*, 349-363.

Keck, C., Bassett, R., and Ludwig, M. (2005). Factors influencing response to ovarian stimulation. Reprod Biomed Online *11*, 562-569.

Kenyon, C. (2001). A conserved regulatory system for aging. Cell *105*, 165-168.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993). A C. elegans mutant that lives twice as long as wild type. Nature *366*, 461-464.

Kiess, W., Reich, A., Meyer, K., Glasow, A., Deutscher, J., Klammt, J., Yang, Y., Muller, G., and Kratzsch, J. (1999). A role for leptin in sexual maturation and puberty? Horm Res *51 Suppl 3*, 55-63.

Kim, R. (2005). Unknotting the roles of Bcl-2 and Bcl-xL in cell death. Biochem Biophys Res Commun *333*, 336-343.

Kirkwood, T. B. (2002). Evolution of ageing. Mech Ageing Dev *123*, 737-745.

Klein, N. A., Battaglia, D. E., Fujimoto, V. Y., Davis, G. S., Bremner, W. J., and Soules, M. R. (1996a). Reproductive aging: accelerated ovarian follicular development associated with a monotropic follicle-stimulating hormone rise in normal older women. J Clin Endocrinol Metab *81*, 1038-1045.

Klein, N. A., Battaglia, D. E., Miller, P. B., Branigan, E. F., Giudice, L. C., and Soules, M. R. (1996b). Ovarian follicular development and the follicular fluid hormones and growth factors in normal women of advanced reproductive age. J Clin Endocrinol Metab *81*, 1946-1951.

Klein, N. A., Battaglia, D. E., Woodruff, T. K., Padmanabhan, V., Giudice, L. C., Bremner, W. J., and Soules, M. R. (2000). Ovarian follicular concentrations of activin, follistatin, inhibin, insulin-like growth factor I (IGF-I), IGF-II, IGF-binding protein-2 (IGFBP-2), IGFBP-3, and vascular endothelial growth factor in spontaneous menstrual cycles of normal women of advanced reproductive age. J Clin Endocrinol Metab *85*, 4520-4525.

Klein, N. A., Houmard, B. S., Hansen, K. R., Woodruff, T. K., Sluss, P. M., Bremner, W. J., and Soules, M. R. (2004). Age-related analysis of inhibin A, inhibin B, and activin a relative to the intercycle monotropic follicle-stimulating hormone rise in normal ovulatory women. J Clin Endocrinol Metab *89*, 2977-2981.

Klein, N. A., Illingworth, P. J., Groome, N. P., McNeilly, A. S., Battaglia, D. E., and Soules, M. R. (1996c). Decreased inhibin B secretion is associated with the monotropic FSH rise in older, ovulatory women: a study of serum and follicular fluid levels of dimeric inhibin A and B in spontaneous menstrual cycles. J Clin Endocrinol Metab *81*, 2742-2745.

Klein, N. A., Soules, M. R., Soules, M. R., Battaglia, D. E., and Klein, N. A. (1998). Endocrine changes of the perimenopause

Inhibin and reproductive aging in women. Clin Obstet Gynecol *41*, 912-920.

Kraemer, D. C., Maqueo, M., Hendrickx, A. G., and Vera Cruz, N. C. (1977). Histology of the baboon endometrium during the menstrual cycle and pregnancy. Fertil Steril *28*, 482-487.

Krey, L., Liu, H., Zhang, J., and Grifo, J. (2001). Fertility and maternal age strategies to improve pregnancy outcome. Ann N Y Acad Sci *943*, 26-33.

Kugu, K., Ratts, V. S., Piquette, G. N., Tilly, K. I., Tao, X. J., Martimbeau, S., Aberdeen, G. W., Krajewski, S., Reed, J. C., Pepe, G. J.*, et al.* (1998). Analysis of apoptosis and expression of bcl-2 gene family members in the human and baboon ovary. Cell Death Differ *5*, 67-76.

Lane, M. A., Baer, D. J., Rumpler, W. V., Weindruch, R., Ingram, D. K., Tilmont, E. M., Cutler, R. G., and Roth, G. S. (1996). Calorie restriction lowers body temperature in rhesus monkeys, consistent with a postulated anti-aging mechanism in rodents. Proc Natl Acad Sci U S A *93*, 4159-4164.

Lane, M. A., Black, A., Handy, A. M., Shapses, S. A., Tilmont, E. M., Kiefer, T. L., Ingram, D. K., and Roth, G. S. (2001). Energy restriction does not alter bone mineral metabolism or reproductive cycling and hormones in female rhesus monkeys. J Nutr *131*, 820-827.

Lane, M. A., Ingram, D. K., Ball, S. S., and Roth, G. S. (1997). Dehydroepiandrosterone sulfate: a biomarker of primate aging slowed by calorie restriction. J Clin Endocrinol Metab *82*, 2093-2096.

Lane, M. A., Ingram, D. K., and Roth, G. S. (1999). Calorie restriction in nonhuman primates: effects on diabetes and cardiovascular disease risk. Toxicol Sci *52*, 41-48.

Lane, M. A., Tilmont, E. M., De Angelis, H., Handy, A., Ingram, D. K., Kemnitz, J. W., and Roth, G. S. (2000). Short-term calorie restriction improves disease-related markers in older male rhesus monkeys (Macaca mulatta). Mech Ageing Dev *112*, 185-196.

Lass, A., and Brinsden, P. (1999). The role of ovarian volume in reproductive medicine. Hum Reprod Update *5*, 256-266.

Lass, A., Skull, J., McVeigh, E., Margara, R., and Winston, R. M. (1997). Measurement of ovarian volume by transvaginal sonography before

ovulation induction with human menopausal gonadotrophin for in-vitro fertilization can predict poor response. Hum Reprod *12*, 294-297.

Le Bourg, E. (2005). [Is caloric restriction a means of delaying ageing and increasing longevity?]. Presse Med *34*, 121-127.

Leidy, L. E., Godfrey, L. R., and Sutherland, M. R. (1998). Is follicular atresia biphasic? Fertil Steril *70*, 851-859.

Li, H., Zeng, Z. H., Wang, L. H., and Wang, J. Q. (2004). [Expression and correlation of apoptosis-related gene c-IAP2 and caspase-4 in sinonasal squamous carcinoma]. Zhonghua Er Bi Yan Hou Ke Za Zhi *39*, 324-327.

Lim, A. S., and Tsakok, M. F. (1997). Age-related decline in fertility: a link to degenerative oocytes? Fertil Steril *68*, 265-271.

Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996). Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell *86*, 147-157.

Lloyd, J. M., Hoffman, G. E., and Wise, P. M. (1994). Decline in immediate early gene expression in gonadotropin-releasing hormone neurons during proestrus in regularly cycling, middle-aged rats. Endocrinology *134*, 1800-1805.

Lu, J. K. H., Hopper, B. R., Vargo, T. M., and Yen, S. S. (1979). Chronological changes in sex steroid, gonadotropin and prolactin secretion in aging female rats desplaying different reproductive states. Biology of Reproduction *21*.

Manabe, N., Goto, Y., Matsuda-Minehata, F., Inoue, N., Maeda, A., Sakamaki, K., and Miyano, T. (2004). Regulation mechanism of selective atresia in porcine follicles: regulation of granulosa cell apoptosis during atresia. J Reprod Dev *50*, 493-514.

Marut, E. L., and Hodgen, G. D. (1982). Antiestrogenic action of high-dose clomiphene in primates pituitary augmentation but with ovarian attenuation. Fertil Steril *38*, 100-104.

Mattison, J. A., Lane, M. A., Roth, G. S., and Ingram, D. K. (2003). Calorie restriction in rhesus monkeys. Exp Gerontol *38*, 35-46.

May-Panloup, P., Chretien, M. F., Malthiery, Y., and Reynier, P. (2004). [Mitochondria and reproduction]. Med Sci (Paris) *20*, 779-783.

Mayer, L. P., Devine, P. J., Dyer, C. A., and Hoyer, P. B. (2004). The follicle-deplete mouse ovary produces androgen. Biol Reprod *71*, 130-138.

Mayer, L. P., Pearsall, N. A., Christian, P. J., Devine, P. J., Payne, C. M., McCuskey, M. K., Marion, S. L., Sipes, I. G., and Hoyer, P. B. (2002). Long-term effects of ovarian follicular depletion in rats by 4-vinylcyclohexene diepoxide. Reprod Toxicol *16*, 775-781.

McCay, C. M., Crowell, M. F., and Maynard, L. A. (1935). The effect of retarded growth upon the length of life span and upon the ultimate body size. J Nutrition *10*, 63-79.

McShane, T. M., and Wise, P. M. (1996). Life-long moderate caloric restriction prolongs reproductive life span in rats without interrupting estrous cyclicity:

effects on the gonadotropin-releasing hormone/luteinizing hormone axis. Biol Reprod *54*, 70-75.

Meredith, S., Dudenhoeffer, G., Butcher, R. L., Lerner, S. P., and Walls, T. (1992). Unilateral ovariectomy increases loss of primordial follicles and is associated with increased metestrous concentration of follicle-stimulating hormone in old rats. Biol Reprod *47*, 162-168.

Merry, B. J. (2004). Oxidative stress and mitochondrial function with aging--the effects of calorie restriction. Aging Cell *3*, 7-12.

Merry, B. J., and Holehan, A. M. (1979). Onset of puberty and duration of fertility in rats fed a restricted diet. J Reprod Fertil *57*, 253-259.

Merry, B. J., and Holehan, A. M. (1985). The endocrine response to dietary restriction in the rat. Basic Life Sci *35*, 117-141.

Messinis, I. E., and Milingos, S. D. (1999). Leptin in human reproduction. Hum Reprod Update *5*, 52-63.

Micevych, P., Sinchak, K., Mills, R. H., Tao, L., LaPolt, P., and Lu, J. K. (2003). The luteinizing hormone surge is preceded by an estrogen-induced increase of hypothalamic progesterone in ovariectomized and adrenalectomized rats. Neuroendocrinology *78*, 29-35.

Miller, P. B., Charleston, J. S., Battaglia, D. E., Klein, N. A., and Soules, M. R. (1999). Morphometric analysis of primordial follicle number in pigtailed monkey ovaries: symmetry and relationship with age. Biol Reprod *61*, 553-556.

Mills, R. H., Romeo, H. E., Lu, J. K., and Micevych, P. E. (2002). Site-specific decrease of progesterone receptor mRNA expression in the hypothalamus of middle-aged persistently estrus rats. Brain Res *955*, 200-206.

Misra, M., Miller, K. K., Almazan, C., Ramaswamy, K., Aggarwal, A., Herzog, D. B., Neubauer, G., Breu, J., and Klibanski, A. (2004). Hormonal and body composition predictors of soluble leptin receptor, leptin, and free leptin index in adolescent girls with anorexia nervosa and controls and relation to insulin sensitivity. J Clin Endocrinol Metab *89*, 3486-3495.

Molskness, T. A., Zelinski-Wooten, M. B., Hild-Petito, S. A., and Stouffer, R. L. (1991). Comparison of the steroidogenic response of luteinized granulosa cells from rhesus monkeys to luteinizing hormone and chorionic gonadotropin. Biol Reprod *45*, 273-281.

Mozo, J., Emre, Y., Bouillaud, F., Ricquier, D., and Criscuolo, F. (2005). Thermoregulation: what role for UCPs in mammals and birds? Biosci Rep *25*, 227-249.

Mozo, J., Ferry, G., Studeny, A., Pecqueur, C., Rodriguez, M., Boutin, J. A., and Bouillaud, F. (2006). Expression of UCP3 in CHO cells does not cause uncoupling, but controls mitochondrial activity in the presence of glucose. Biochem J *393*, 431-439.

Nagai, S., Mabuchi, T., Hirata, S., Shoda, T., Kasai, T., Yokota, S., Shitara, H., Yonekawa, H., and Hoshi, K. (2004). Oocyte mitochondria: strategies to improve embryogenesis. Hum Cell *17*, 195-201.

Nappi, R. E., Sinforiani, E., Mauri, M., Bono, G., Polatti, F., and Nappi, G. (1999). Memory functioning at menopause: impact of age in ovariectomized women. Gynecol Obstet Invest *47*, 29-36.

Navot, D., Bergh, P. A., Williams, M. A., Garrisi, G. J., Guzman, I., Sandler, B., and Grunfeld, L. (1991). Poor oocyte quality rather than implantation failure as a cause of age-related decline in female fertility. Lancet *337*, 1375-1377.

Navot, D., Drews, M. R., Bergh, P. A., Guzman, I., Karstaedt, A., Scott, R. T., Jr., Garrisi, G. J., and Hofmann, G. E. (1994). Age-related decline in female fertility is not due to diminished capacity of the uterus to sustain embryo implantation. Fertil Steril *61*, 97-101.

Nelson, J. F., Gosden, R. G., and Felicio, L. S. (1985). Effect of dietary restriction on estrous cyclicity and follicular reserves in aging C57BL/6J mice. Biol Reprod *32*, 515-522.

Nelson, J. F., Karelus, K., Bergman, M. D., and Felicio, L. S. (1995). Neuroendocrine involvement in aging: evidence from studies of reproductive aging and caloric restriction. Neurobiol Aging *16*, 837-843; discussion 855-836.

Nesse, R. M., and Williams, G. C. (1996). Why we get sick: The new science of Darwinian medicine (New York: Vintage Books).

Nichols, S. M., Bavister, B. D., Brenner, C. A., Didier, P. J., Harrison, R. M., and Kubisch, H. M. (2005). Ovarian senescence in the rhesus monkey (Macaca mulatta). Hum Reprod *20*, 79-83.

Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell *74*, 609-619.

Packer, C., Tatar, M., Collins, A., Blurton Jones, N. G., Hawkes, K., and O'Connell, J. F. (1998). Reproductive cessation in female mammals

Antiquity of postreproductive life: are there modern impacts on hunter-gatherer postreproductive life spans? Nature *392*, 807-811.

Park, S. J., Goldsmith, L. T., Reinert, A., Skurnick, J. H., Castracane, V. D., and Weiss, G. (2000). Perimenopausal women are deficient in an estrogen positive feedback on LH secretion mechanism (abstract), Paper presented at: Program of the 82nd annual meeting of the International Endocrine Society.

Peccei, J. S. (2001). A critique of the grandmother hypotheses: old and new. Am J Hum Biol *13*, 434-452.

Penarrubia, J., Fabregues, F., Manau, D., Creus, M., Casals, G., Casamitjana, R., Carmona, F., Vanrell, J. A., and Balasch, J. (2005). Basal and stimulation day 5 anti-Mullerian hormone serum concentrations as predictors of ovarian response and pregnancy in assisted reproductive technology cycles stimulated with gonadotropin-releasing hormone agonist--gonadotropin treatment. Hum Reprod *20*, 915-922.

Perez, G. I., Robles, R., Knudson, C. M., Flaws, J. A., Korsmeyer, S. J., and Tilly, J. L. (1999). Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency. Nat Genet *21*, 200-203.

Prior, J. C. (1998). Perimenopause: the complex endocrinology of the menopausal transition. Endocr Rev *19*, 397-428.

Prior, J. C. (2005). Ovarian aging and the perimenopausal transition: the paradox of endogenous ovarian hyperstimulation. Endocrine *26*, 297-300.

Reame, N. E., Wyman, T. L., Phillips, D. J., de Kretser, D. M., and Padmanabhan, V. (1998). Net increase in stimulatory input resulting from a decrease in inhibin B and an increase in activin A may contribute in part to the rise in follicular phase follicle-stimulating hormone of aging cycling women. J Clin Endocrinol Metab *83*, 3302-3307.

Roberts, V. J., Barth, S., el-Roeiy, A., and Yen, S. S. (1993). Expression of inhibin/activin subunits and follistatin messenger ribonucleic acids and proteins in ovarian follicles and the corpus luteum during the human menstrual cycle. J Clin Endocrinol Metab *77*, 1402-1410.

Robertson, D. M., and Burger, H. G. (2002). Reproductive hormones: ageing and the perimenopause. Acta Obstet Gynecol Scand *81*, 612-616.

Roth, G. S., Ingram, D. K., and Lane, M. A. (1999). Calorie restriction in primates: will it work and how will we know? J Am Geriatr Soc *47*, 896-903.

Sakamaki, K., Yoshida, H., Nishimura, Y., Nishikawa, S., Manabe, N., and Yonehara, S. (1997). Involvement of Fas antigen in ovarian follicular atresia and luteolysis. Mol Reprod Dev *47*, 11-18.

Santoro, N. (2005). The menopausal transition. Am J Med *118*, 8-13.

Sasson, R., Rimon, E., Dantes, A., Cohen, T., Shinder, V., Land-Bracha, A., and Amsterdam, A. (2004). Gonadotrophin-induced gene regulation in human

granulosa cells obtained from IVF patients. Modulation of steroidogenic genes, cytoskeletal genes and genes coding for apoptotic signalling and protein kinases. Mol Hum Reprod *10*, 299-311.

Scarbrough, K., and Wise, P. M. (1990). Age-related changes in pulsatile luteinizing hormone release precede the transition to estrous acyclicity and depend upon estrous cycle history. Endocrinology *126*, 884-890.

Scheffer, G. J., Brockmans, F. J., Dorland, M., Loorman, C. W., Blankenstein, M. A., and teVelde, E. R. (1998). The number of antral follicles measured by transvaginal ultrasound (TVS) is the best predictor of reproductive age in a group of normal fertile women. Hum Reprod *13*, 78.

Schreiber, S. N., Emter, R., Hock, M. B., Knutti, D., Cardenas, J., Podvinec, M., Oakeley, E. J., and Kralli, A. (2004). The estrogen-related receptor alpha (ERRalpha) functions in PPARgamma coactivator 1alpha (PGC-1alpha)-induced mitochondrial biogenesis. Proc Natl Acad Sci U S A *101*, 6472-6477.

Schreiber, S. N., Knutti, D., Brogli, K., Uhlmann, T., and Kralli, A. (2003). The transcriptional coactivator PGC-1 regulates the expression and activity of the orphan nuclear receptor estrogen-related receptor alpha (ERRalpha). J Biol Chem *278*, 9013-9018.

Sedlak, T. W., Oltvai, Z. N., Yang, E., Wang, K., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995). Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. Proc Natl Acad Sci U S A *92*, 7834-7838.

Serna, J., and Garcia-Velasco, J. A. (2005). Effect of repeated assisted reproduction techniques on the ovarian response. Curr Opin Obstet Gynecol *17*, 233-236.

Sherman, P. W. (1998). The evolution of menopause. Nature *392*, 759, 761.

Sherwin, B. B. (2003). Estrogen and cognitive functioning in women. Endocr Rev *24*, 133-151.

Shideler, S. E., Gee, N. A., Chen, J., and Lasley, B. L. (2001). Estrogen and progesterone metabolites and follicle-stimulating hormone in the aged macaque female. Biol Reprod *65*, 1718-1725.

Sinclair, D. A. (2005). Toward a unified theory of caloric restriction and longevity regulation. Mech Ageing Dev *126*, 987-1002.

Slot, K. A., de Boer-Brouwer, M., Houweling, M., Vaandrager, A. B., Dorrington, J. H., and Teerds, K. J. (2006). Luteinizing hormone inhibits Fas-induced apoptosis in ovarian surface epithelial cell lines. J Endocrinol *188*, 227-239.

Smith, J. V., Heilbronn, L. K., and Ravussin, E. (2004). Energy restriction and aging. Curr Opin Clin Nutr Metab Care *7*, 615-622.

Sole, C., Dolcet, X., Segura, M. F., Gutierrez, H., Diaz-Meco, M. T., Gozzelino, R., Sanchis, D., Bayascas, J. R., Gallego, C., Moscat, J., *et al.* (2004). The death receptor antagonist FAIM promotes neurite outgrowth by a mechanism that depends on ERK and NF-kapp B signaling. J Cell Biol *167*, 479-492.

Soules, M. R., Battaglia, D. E., and Klein, N. A. (1998). Inhibin and reproductive aging in women. Maturitas *30*, 193-204.

Speroff, L., Glass, R. H., and Kase, N. G. (1999). Clinical Gynecologic Endocrinology and Infertility, 6 edn: Lippincott, William and Wilkins).

Springer, L. N., Flaws, J. A., Sipes, I. G., and Hoyer, P. B. (1996). Follicular mechanisms associated with 4-vinylcyclohexene diepoxide-induced ovotoxicity in rats. Reprod Toxicol *10*, 137-143.

Syrop, C. H., Willhoite, A., and Van Voorhis, B. J. (1995). Ovarian volume: a novel outcome predictor for assisted reproduction. Fertil Steril *64*, 1167-1171.

Taylor, A. E., Hubbard, J., and Anderson, E. J. (1999). Impact of binge eating on metabolic and leptin dynamics in normal young women. J Clin Endocrinol Metab *84*, 428-434.

te Velde, E. R., Scheffer, G. J., Dorland, M., Broekmans, F. J., and Fauser, B. C. (1998). Developmental and endocrine aspects of normal ovarian aging. Mol Cell Endocrinol *145*, 67-73.

Themmen, A. P. (2005). Anti-Mullerian hormone: its role in follicular growth initiation and survival and as an ovarian reserve marker. J Natl Cancer Inst Monogr, 18-21.

Thomas, F. H., Ethier, J. F., Shimasaki, S., and Vanderhyden, B. C. (2005). Follicle-stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors. Endocrinology *146*, 941-949.

Tilly, J. L., Kowalski, K. I., Schomberg, D. W., and Hsueh, A. J. (1992). Apoptosis in atretic ovarian follicles is associated with selective decreases in messenger ribonucleic acid transcripts for gonadotropin receptors and cytochrome P450 aromatase. Endocrinology *131*, 1670-1676.

Tilly, J. L., Tilly, K. I., Kenton, M. L., and Johnson, A. L. (1995). Expression of members of the bcl-2 gene family in the immature rat ovary: equine chorionic gonadotropin-mediated inhibition of granulosa cell apoptosis is associated with decreased bax and constitutive bcl-2 and bcl-xlong messenger ribonucleic acid levels. Endocrinology *136*, 232-241.

Tran, H., Brunet, A., Griffith, E. C., and Greenberg, M. E. (2003). The many forks in FOXO's road. Sci STKE *2003*, RE5.

Treloar, A. E. (1981). Menstrual cyclicity and the pre-menopause. Maturitas *3*, 249-264.

Tremellen, K. P., Kolo, M., Gilmore, A., and Lekamge, D. N. (2005). Anti-mullerian hormone as a marker of ovarian reserve. Aust N Z J Obstet Gynaecol *45*, 20-24.

van Rooij, I. A., Broekmans, F. J., te Velde, E. R., Fauser, B. C., Bancsi, L. F., de Jong, F. H., and Themmen, A. P. (2002). Serum anti-Mullerian hormone levels: a novel measure of ovarian reserve. Hum Reprod *17*, 3065-3071.

Ventura, S. J., Abma, J. C., Mosher, W. D., and Henshaw, S. (2004). Estimated pregnancy rates for the United States, 1990-2000: an update. Natl Vital Stat Rep *52*, 1-9.

Visser, J. A., de Jong, F. H., Laven, J. S., and Themmen, A. P. (2006). Anti-Mullerian hormone: a new marker for ovarian function. Reproduction *131*, 1-9.

Vollman, R. F. (1977). The Menstrual Cycle. (Philadelphia: WB Saunders).

vom Saal, F. S., Finch, C. E., and Nelson, J. F. (1994). Natural history and mechanisms of reproductive aging in humans, In Physiology of Reproduction, E. Knobil, and J. Neill, eds. (New York: Raven Press), pp. 861-1010.

Wassarman, P. M. (1994). The mammalian ovum, In Physiology of Reproduction, E. Knobil, and J. Neill, eds. (New York: Raven Press), pp. 79-122.

Welt, C. K., McNicholl, D. J., Taylor, A. E., and Hall, J. E. (1999). Female reproductive aging is marked by decreased secretion of dimeric inhibin. J Clin Endocrinol Metab *84*, 105-111.

Weston, A. M., Zelinski-Wooten, M. B., Hutchison, J. S., Stouffer, R. L., and Wolf, D. P. (1996). Developmental potential of embryos produced by in-vitro fertilization from gonadotrophin-releasing hormone antagonist-treated macaques stimulated with recombinant human follicle stimulating hormone alone or in combination with luteinizing hormone. Hum Reprod *11*, 608-613.

Williams, G. C. (1957). Pleiotropy, natural selection, and the evolution of senescence. Evolution *11*, 389-411.

Williams, N. I., Helmreich, D. L., Parfitt, D. B., Caston-Balderrama, A., and Cameron, J. L. (2001). Evidence for a causal role of low energy availability

in the induction of menstrual cycle disturbances during strenuous exercise training. J Clin Endocrinol Metab *86*, 5184-5193.

Willy, P. J., Murray, I. R., Qian, J., Busch, B. B., Stevens, W. C., Jr., Martin, R., Mohan, R., Zhou, S., Ordentlich, P., Wei, P.*, et al.* (2004). Regulation of PPARgamma coactivator 1alpha (PGC-1alpha) signaling by an estrogen-related receptor alpha (ERRalpha) ligand. Proc Natl Acad Sci U S A *101*, 8912-8917.

Wise, P. M. (1991). Neuroendocrine influences on aging of the female reproductive system. Frontiers in Neuroendocrinology *12*, 323-356.

Wu, J. M., Takahashi, D. L., Lawson, M. S., Pau, F., Mattison, J. A., Ingram, D. K., Roth, G. S., Lane, M. A., Ottinger, M. A., and Zelinski-Wooten, M. B. (2004). Spontaneous and gonadotropin-stimulated ovarian function during short-term moderate caloric restriction in macaques, Paper presented at: XVth Ovarian Workshop (Vancouver, Canada).

Wu, J. M., Zelinski, M. B., Ingram, D. K., and Ottinger, M. A. (2005). Ovarian aging and menopause: current theories, hypotheses, and research models. Exp Biol Med (Maywood) *230*, 818-828.

Yang, E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., and Korsmeyer, S. J. (1995). Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell *80*, 285-291.

Zeleznik, A. J., Midgley, A. R., Jr., and Reichert, L. E., Jr. (1974). Granulosa cell maturation in the rat: increased binding of human chorionic gonadotropin

following treatment with follicle-stimulating hormone in vivo. Endocrinology *95*, 818-825.

Zelinski-Wooten, M. B., Hutchison, J. S., Hess, D. L., Wolf, D. P., and Stouffer, R. L. (1995). Follicle stimulating hormone alone supports follicle growth and oocyte development in gonadotrophin-releasing hormone antagonist-treated monkeys. Hum Reprod *10*, 1658-1666.

Zhang, L., Weston, A. M., Denniston, R. S., Goodeaux, L. L., Godke, R. A., and Wolf, D. P. (1994). Developmental potential of rhesus monkey embryos produced by in vitro fertilization. Biol Reprod *51*, 433-440.