OVULATION IN SAIMIRI SCIUREUS: INDUCTION, DETECTION AND INHIBITION

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ABSTRACT

OVULATION IN *SAIMIRI SCIUREUS:* INDUCTION, DETECTION AND INHIBITION

By

Richard Miller Harrison

This series of studies was conducted to determine the suitability of *Saimiri sciureus* as a nonhuman primate research model in studies involving the induction, detection and inhibition of ovulation. This species repeatedly ovulated in response to a regime of exogenous hormones. A pretreatment of progesterone followed by follicle stimulating hormone (FSH) and human chorionic gonadotropin (HCG) usually stimulated single ovulations 6 to 14 hours after the HCG injection.

S. sciureus proved to be an excellent model for the laparoscopic examination of ovulation. The ovaries may be continuously observed for a 6-hour period and examinations can be repeated at 24-hour intervals with no detrimental effects. Monkeys repeatedly subjected to laparoscopy over a 3-year period appeared healthy and reproductively normal.

Ovulation can be controlled by subcutaneous injections of megestrol acetate. Doses of 500 µg/day given concomitantly with and 1 day after the FSH blocked the ovulation whereas 50 µg/day had no effect on the ovulatory

response to the gonadotropins. Intermediate levels gave dose related but nonlinear results.

Megestrol acetate administered to *S. sciureus* via subcutaneous silastic implants also influenced ovulation. This influence was related to the duration of time between insertion and start of the ovulation induction scheme. The ovulatory response was influenced by the elimination of the progesterone pretreatment in monkeys with silastic implants containing megestrol acetate.

The mean amount of MA released daily from silastic implants *in vivo* was significantly greater than the amount released from capsules incubated *in vitro* (56.3 µg compared to 12.2 µg). The *in vitro* release rate was not constant over a 34-day period.

The megestrol acetate released *in vivo* accumulated in the female reproductive tract with highest concentrations in the oviducts and ovaries.

S. sciureus maintained in a stable laboratory environment showed a seasonal response to the ovulation induction regime. Approximately 1.5 years were required before the monkeys began to show a regular pattern to their response. Fully acclimated monkeys showed a minimal ovulatory response during periods of highest relative humidity with maximal response in the middle of the dry season. The environmental stimulus appeared to be changes in relative humidity rather than absolute humidity.

OVULATION IN *SAIMIRI SCIUREUS:* INDUCTION, DETECTION AND INHIBITION

By

Richard Miller Harrison

A THESIS

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To my friend,

companion and wife

Joanna

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INTRODUCTION

"During the past two decades, there has been increasing interest in the use of nonhuman primates for studies that are directly applicable to problems in human reproduction and that will have a predictive value" (Diczfalusy and Standley, 1972).

The above quotation emphasizes the importance of nonhuman primates as models for physiologic studies relating to humans. The use of nonhuman primates to study infectious pathology in man has been widespread because so many human infectious diseases can be produced in similar forms in nonhuman primates. The reproductive similarities between human females and females of the Old World nonhuman primate species, especially *Macaca*, are quite notable. Both have menstrual cycles of approximately 28 days, most commonly ovulate one ovum at about mid-cycle, implant in a simplex uterus about 6 days after ovulation and will accept males at any time during their cycle but with a greater interest at mid-cycle. The extensive use of *Macaca mulatta* in ovulation studies has been documented in a review by van Wagenen (1966).

Ovulation was induced in nonhuman primates as early as 1935 by Hisaw, Greep and Fevold using crude ovine anterior pituitary extracts. These procedures have been refined and elaborated. Today ovulation can be induced in macaques using a number of hormonal regimes and agents.

The combination of physiological similarities and procedural techniques makes the nonhuman primate an excellent model for research in many areas of human reproduction. The only drawbacks to the use of such models are those of economics, availability and ease of handling. Although laboratory-born primates have advantages over wild-caught animals, the limited availability of such animals restricts their use in research at this time.

Many of these difficulties are overcome by using the smaller New World monkeys. *Saimiri sciureus* is now the second most widely used nonhuman primate in the United States. The advantages of *S. sciureus* are generally due to its size and its abundance in its natural habitat. Because large numbers can be trapped and shipped to this country they are less expensive than Old World monkeys. They are less susceptible to tuberculosis, require simpler caging and do not appear to be as stressed by simple surgical procedures. A full grown male *S. sciureus* weighs about one kg compared to 7 to 10 kg for *M. mulatta*. One individual can easily handle an alert squirrel monkey and do most

procedural techniques without assistance. One of the more important considerations, in light of the increased interest in conservation, is that *S. sciureus* is not at present on the endangered species list.

Saimiri sciureus is dissimilar, in some aspects of its reproductive physiology, to humans. It does not have an overt menstrual cycle and is a seasonal breeder. Because of its advantages as a nonhuman primate research subject, the present study was undertaken to determine the suitability of *Saimiri sciureus* as a model in studies involved in the induction, detection and inhibition of ovulation.

The objectives of these investigations were:

1. to develop the techniques necessary to detect and photograph ovulation in *Saimiri sciureus*, with minimal trauma to the animal;
2. to study acclimation and the effects of seasonal environmental stimuli on the ability of *Saimiri sciureus* to ovulate in response to exogenous hormones when maintained in a laboratory environment;
3. to determine the value of this species as a research model in ovulation studies by ascertaining its response to a progestin currently being studied as a low dose, oral contraceptive agent.

LITERATURE REVIEW

To fully understand the current knowledge concerning the induction and inhibition of ovulation in *Saimiri sciureus*, it is first necessary to learn about the natural occurrence of ovulation. When nonhuman primates are in the wild, seminatural environments, or breeding colonies, the ovulation is usually detected by indirect methods. Most commonly the time of copulation is extrapolated from the date of birth or estimated age of young. Successful coitus (resulting in birth of a young) is the most positive proof of ovulation in the wild.

The breeding season is defined as the period of time during which all breeding takes place. A breeding peak, in contrast, is a period of time during which most breeding takes place. The former term is inclusive whereas the latter is not.

Ideally, field studies observe the seasonal distribution of copulation, sex skin changes, and births and should meet certain criteria (Lancaster and Lee, 1965):

1. observations should be on free-ranging populations, in their natural habitat, eating natural foods;

2. observations should be both long termed and continuous;
3. birth dates, time of copulation, and age of young should be estimated as accurately as possible;
4. the population observed should be as large as possible.

No previous study meets all of these criteria, yet several have contributed to our understanding of nonhuman primate reproductive cycles and the effects of seasonal climatic changes on these cycles.

Natural Ovulation-Seasonality

Macaca sylvana (Barbary Apes) on Gibraltar are the second most northern species of nonhuman primates. The climate at Gibraltar is dry and hot in the summer and mild and rainy in the winter. Rainfall is rare except between September and May. The temperature ranges from 27°C in August to 10°C in January. Fig trees fruit on Gibraltar during the spring and early summer and fruit is freely eaten by the monkeys. The majority of their diet comes from city garbage and occasional provisions. Records on these monkeys were not consistently kept from the 1920's until early 1940's. Since that time, however, all births have been recorded. A six-month study was conducted in mid-1964 to observe mating behavior and the female reproductive cycle (MacRoberts and MacRoberts, 1966). Of 147 births recorded,

145 occurred from May to September. Only during November were mountings observed to result in ejaculation. Female sex swelling was also noted in November. The late gestation and early post-natal period coincides with the fruiting season.

Koford (1963) reported that *Macaca mulatta* on Cayo Santiago, in the Caribbean, have a mating season from July to January. The monkeys in this colony are descended from stock transferred from India in 1938 by C. R. Carpenter. The *Macaca* are free-ranging and are provided with supplemental food.

Vandenbergh and Vessey (1968) reported that free-ranging *Macaca mulatta* on two islands, La Cueva and Guayacan, near Puerto Rico, demonstrate seasonal rhymicity in mating activity. From 1964 to 1966 all births occurred between March and August and 74% occurred during May and June. The peak mating activity was in November, December and January, following the peak rainfall in August to October. These workers concluded that a correlation between rainfall and mating activity appears to be present in these monkeys as well as those at Cayo Santiago and those in the natural habitat of Northern India. The effects of the rainfall were believed to be mediated through vegetational changes.

The reproductive cycle of *Cercopithecuss mitis kolbi* was investigated by Omar and DeVos (1971). Conceptions were

found to be confined to a discrete season from July to November with a peak in October. This period coincided with the dry period. Births were confined to a period from November to March with more than one-third during the last month. Lactation occurred during the wet season. Omar and DeVos quoted previous workers who reported reproductive behavior in *Macaca fuscata*, *Macaca radiata*, *Presbytis entellus*, *Papio hamadryas* and *Papio cynocephalus*.

Ateles belzebuth, a South American spider monkey, has been reported to have no seasonal birth peak. Estimated birth dates of feral *A. belzebuth* occurred in September through January with some births in April (Klein, 1971).

The cycles may change when nonhuman primates are held in captive environments. The birth periods for spider monkeys in a San Francisco colony ranges from August to May (Klein, 1971). *Macaca mulatta* in a New York colony had the greatest number of conceptions between September and November. The stress of a translocation of this colony to a new location 40 miles away resulted in an 8-month delay of this seasonal peak (Niemann, 1971).

Riesen, Meyer and Wolf (1971) found that many menstrual cycles during the summer are anovulatory. *Macaca mulatta*, maintained in an indoor colony with controlled light cycles and constant temperature, ovulated in 5 of 24 cycles from June to August. These same monkeys ovulated

in 30 of 31 cycles from December to February. This difference was significant ($p < 0.001$).

Feral troops of *Macaca fuscata* have a 5- to 6-month fall mating season. *M. fuscata* maintained at the Oregon Regional Primate Research Center have a mating season that ranges from July to February. The occurrence of mating is influenced by the presence of infant monkeys with the females (Eaton, 1972).

A recent report on artificial troops of free-ranging monkeys indicate that peak birth seasons occur with *M. fuscata fuscata* in March to April, with *M. fuscata yakui* in May, and with *M. iris* in April to June. *Papio anubis* at Taukumi Island have no particular birth season (Nomura *et al.*, 1971).

Macaca mulatta, in an indoor breeding colony in New York, do not produce pregnancies evenly throughout the year. Peak fertility occurs from December to February with 69 of 96 pregnancies in a 3-year period occurring during this period (Segal *et al.*, 1971). These workers indicated that seasonality is experienced in other colonies as well. The male does not contribute to the seasonal infertility since high sperm concentrations are found in ejaculates during periods of both high and low conception rates.

Relatively few field studies have been made on *Saimiri sciureus*. Baldwin and Baldwin (1971) stated that

prior to their 1968 and 1969 studies, only reports by Thorington (1968) and Sanderson (1957) had been published on the feral *Saimiri*. Thorington reported on a troop of *Saimiri sciureus* consisting of 3 males, 5 females and 10 juveniles in an area near San Martin, Colombia. The birth of 4 infants during February and early March and the late pregnancy appearance of the fifth female established a birth season for this troop. Rosenblum (1968) states that probably the normal birth season of *S. sciureus* in the Amazon basin ranges from late December to early March with a peak in January. Baldwin and Baldwin (1971) found that the birth season in Panama was in June and July. Their observations in the Ilanos area (approximately the same area as Thorington's study) supported the birth season described above. Infants observed during June to September in the area were 5 to 7 months of age, suggesting a birth season in February. Assuming a 5.5 month gestation period, the mating season in Colombia must occur in July. Baldwin and Baldwin (1971) confirmed this, as they observed behavioral patterns associated with mating in *S. sciureus*.

A unique environment for the observation of reproductive behavior in *Saimiri sciureus* was established in 1960 at the Monkey Jungle, south of Miami, Florida. Complete records of reproduction have been kept there. DuMond and Hutchinson (1967) reported on two aspects of seasonal

reproduction in *S. sciureus* living in this seminatural environment. Although all births were confined to very discrete periods each year this period shifted from winter to summer in 3 years. After this acclimation period the birth season has stabilized and females born in the colony give birth in phase with the older adults. The males in this colony begin to develop a "fatted" appearance at the approach of the mating season. This condition is characterized by increased body weight and increased fluffiness of pelage especially around the upper body. During the "fatted" phase active spermatogenesis is evident in nearly all seminiferous tubules whereas during the "non-fatted" phase the tubules consist essentially of a basal layer of spermatogonia and Sertoli cells with enlarged lumena devoid of cells. DuMond and Hutchinson (1967) claim a positive correlation between environmental precipitation cycles and reproductive cycles. They suggest the mediation may be through changes in solar radiation or nutritional factors relating to forest flowering.

Lehner *et al.* (1967) observed no reproductive activity in a troop of *Saimiri sciureus* trapped near Teresina, Brazil, during its first 1.5 years of captivity. These monkeys were maintained in an indoor-outdoor facility in North Carolina. Of the 17 births reported at the end of this acclimation period, 16 occurred between June 4 and

July 15, the last occurred on September 3. Another group of *S. sciureus* received in December 1964 showed no reproductive activity until February 1966. Thirty-three females became pregnant and 29 births occurred between July 15 and September 14. The other 4 births were in October. These workers suggest that at least 12 to 18 months are necessary for the monkeys to acclimate to the laboratory.

A similar acclimation period was necessary for *Saimiri sciureus* in an outdoor cage in southern Florida (Taylor, 1968). One male mated with three females during the winter with births taking place in May and June. These monkeys had been in the facility for approximately 1 year prior to mating.

The monkey Jungle *S. sciureus* colony was observed in 1966 and 1967 to study male behavior (Baldwin, 1968). The reproductive cyclicity was hypothesized to be triggered by seasonal rainfall or by a phenomenon dependent on rainfall, such as vegetative growth or solar radiation. During the birth season the males were inactive socially, were quiet and remained apart from the females. In the mating season they were hyperactive, highly excitable, aggressive and vocal. They frequently engaged in penile display. Interestingly, most mating was by subdominant males since they were quieter and attracted less attention when they approached receptive females.

The above references on reproduction of *Saimiri sciureus* refer to either feral monkeys or those maintained in cages with outdoor exposure. The remaining references in this section concern observations made on monkeys in controlled laboratory colonies with minimal environmental changes.

A period of at least 9 months in a stable group has been suggested for successful mating in a laboratory of *S. sciureus* (Bowden, Winter and Ploog, 1967). Hopf (1967) found that a minimum of 15 months and an average of 27 months was required between arrival and date of first parturition. All births (11 in 3 years) took place from July to November. Lang (1967) reported that *S. sciureus* bred in his laboratory gave birth during August or September.

The *S. sciureus* colony at the laboratories of British Drug Houses has a stable environment with a temperature of 24° ± 2°C, a relative humidity of 55% and a 15-hr light/9-hr dark cycle. No significant shift in birth season was noted in this colony with births from November to January. The "fatted" male phenomenon was observed. Successful mating did not occur until the second season of captivity (Bantin, 1969).

In a recent review on reproduction of *S. sciureus* in the laboratory, Rosenblum (1973) states that successful

breeding programs must take into account the limited breeding periods available. He reports that the "fatting" of the males is testosterone dependent and emphasized the importance of male-female synchronization. Vaginal estrus, as determined by a predominance of cornified epitheal cells in a smear (Rosenblum *et al.*, 1967), was maximal in May, June and July of the first year with births in December and January. In the last 5 years this seasonality has flattened out and now full term parturition and viable births have occurred in every month. Rosenblum (1973) suggests that monkeys received at different times should not be mixed for mating as each sex may influence the reproductive activity of the other sex.

Induced Ovulation

Hisaw, Greep and Fevold (1935) reported follicular development, with occasional ovulation, following injection of crude and purified pituitary extracts in *Macaca mulatta*. Follicular development and sporadic ovulation in adult macaques were obtained using gonadotropins in the early 1940's (Hartman, 1942). Induced ovulation in monkeys always resulted in superovulation (van Wagenen and Simpson, 1957). Simpson and van Wagenen (1958) induced ovulation in immature and adult *Macaca* with pituitary preparations. Monkey anterior pituitary preparations regularly induced

ovulation. Injections were given from Day 5 or 6 to mid-cycle. Multiple ovulations resulted in mature monkeys.

Hypophysectomized *M. mulatta* were used to study the effects of desiccated thyroid, porcine follicle stimulating hormone (FSH) and human chorionic gonadotropin (HCG) on ovulation (Knobil, Kostyo and Greep, 1959). These studies eliminated the effects of endogenous gonadotropin secretion by removing the pituitary source. They found that atrophic ovaries readily responded to FSH, FSH preparations of non-primate origin stimulated follicular growth, excessive stimulation blocked ovulation and best results were obtained with small doses of FSH ($\leq$ 5 units/day) and large doses of HCG ($\geq$ 1000 units/day).

Multiple ovulations were induced in adult *M. mulatta* following injections of human postmenopausal gonadotropin (HMG) and HCG (Simpson and van Wagenen, 1962). Other workers using these gonadotropins found no significant differences in birth rates when induced ovulation was followed by coitus or artificial insemination compared to birth rates as a result of spontaneous ovulation followed by copulation or artificial insemination (Dede and Plentl, 1966).

A review of ovulation induction in *Macaca mulatta* has been presented by van Wagenen (1968) covering 25 years of work.

Wan and Balin (1969) used HMG and HCG, clomiphene citrate, and dl-18-methyl estriol to induce ovulation in adult *M. mulatta*. They were successful in obtaining high incidences of single ovulations.

A recent publication reports the successful induction of ovulation in amenorrheic *M. mulatta* using various FSH/LH ratios in combination with HCG or human pituitary LH (Breckwoldt, Bettendorf and Garciá, 1971).

No attempts to induce ovulation in a New World species of monkey were reported prior to Bennett's studies in 1967. He injected *S. sciureus* with pregnant mares serum gonadotropin (PMS) for 9 days and HCG for the last 4 days. Ovulation appeared to be multiple when it occurred (Bennett, 1967a). He demonstrated that ova could be recovered from the oviducts, but that high concentrations of PMS hastened tubal transport due to increased ovarian estrogen (1967b).

Dukelow (1970) used FSH, PMS and HMG for follicular development and HCG to induce ovulation. He was able to induce timed single ovulations, and to recover ova from some monkeys.

Ovulation was induced in immature squirrel monkeys with PMS and HCG (Fajer and Bechini, 1971). Extensive follicular growth was noted and ovulation detected histologically. Detectable amounts of pregnenolone and progesterone were found in the ovarian vein blood after

2 days of treatment. The interstitial tissue was suggested as the source of pregnenolone.

These studies in *S. sciureus* suggested the value of this species as a nonhuman primate model for ovulation induction studies.

Inhibition of Ovulation

The difficulty of detecting ovulation in primates has precluded many studies on the inhibition of ovulation. Goisis (1964) reported that ovulation could be blocked in *Papio* by injections of 30 mg medroxyprogesterone acetate, progesterone or the cyclopentyl enol ether of 17 α-acetoxy-progesterone. The mechanism of action of such progestogens was suggested by Spies and Niswender (1972) who found that 0.5 to 5.0 mg doses of progesterone administered to adult *M. mulatta* on Days 9 to 18 blocked the preovulatory LH surge and ovulation.

Ovulation can be blocked in women with 5 mg daily of megestrol acetate (MA, 17 α-acetoxy-6-methylpregna-4,6-diene-3,20 dione) (Suran, 1967; Starup and Lebech, 1967). The antifertility action of MA can be isolated from the antiovulatory action by administration of low doses ($\leq$ 0.5 mg).

The importance of the mode of administration of these contraceptive steroids has been recently emphasized in a discussion by Goldzieher and Kraemer (1972).

The use of silicone rubber (Silastic) capsules containing steroids as a means of continuously administering the steroids to test subjects was first described by Dziuk and Cook (1966). They reported that *in vitro* release was constant for several days in capsules containing estradiol, progesterone or 17 α-acetoxy-6-methyl-16 methylenepregna-4, 6-diene-3,20-dione (melengestrol acetate, MGA). Implants containing the MGA were placed subcutaneously in ewes. The incidence of heat was reduced while the implants were *in situ*. Pharriss and Hendrix (1969) reported that medroxy-progesterone acetate (MPA), administered via a subcutaneous implant, provided adequate contraceptive control with elimination of the daily rise and fall of serum MPA concentrations seen with oral dosing and with ease of reversibility.

The biological activity of MA administered to rats via subcutaneous dimethylpolysiloxane (Silastic) implants has been reported to be 6 to 25 times more potent than that administered by either subcutaneous injections or oral gavage (Chang and Kincl, 1968).

Megestrol acetate can be an effective contraceptive agent when administered to women via subcutaneous silastic implants. Approximately 90% of the cycles are ovulatory and the site(s) of action is peripheral to the ovary (Croxatto *et al.*, 1969). There are no local or systemic complications

(Tatum *et al.*, 1969). The *in vitro* diffusion of MA from silastic implants was reported by Benagiano *et al.* (1970) to be 16.0 ± 0.04 µg/24 hrs for Days 10 to 20 and 16.2 ± 0.03 µg/24 hrs for Days 21 to 40 of incubation. They did not consider *in vivo* release to be constant. In a long term human study no pregnancies occurred in over 1400 cycles in women with subcutaneous silastic capsules containing MA (Coutinho *et al.*, 1970).

A study in castrated *M. mulatta* indicated that MA was 10 times more potent by implant than when administered orally. Progestational changes in the estrogen primed endometria and a dose related infiltration of lymphocytes into the endometrium were observed (Cuadros, Brinson and Sundaram, 1970).

The contributions of these studies to a better understanding of ovulation, its induction and inhibition, have been great. They indicate that factors not easily controlled in a laboratory environment may influence the occurrence of ovulation. The apparent differences noted by various workers may be due to these environmental influences. Until recently no techniques existed for long term study of ovulation in a laboratory nonhuman primate.

MATERIALS AND METHODS

Subjects

All monkeys used in these studies were squirrel monkeys *(Saimiri sciureus)* of the Brazilian type as described by Cooper (1968). They were purchased from Tarpon Zoo Inc., Tarpon Springs, Florida, and were quarantined for at least 30 days between import into this country and time of sale. All monkeys were mature females weighing between 550 and 950 grams at the time of purchase.

When received at our colony the monkeys were weighed, given an identification tag and a prophylactic injection of Procaine Penicillin G. (W. A. Butler Co., Columbus, Ohio, 300,000 units per ml, 0.5 ml per animal, intramuscularly). The monkeys were placed in groups of five in stainless steel flush type cages, 0.6 m x 0.7 m x 0.8 m. These cages were rinsed with clean water daily.

Newly arrived squirrel monkeys were fed a mixture of crushed Wayne Monkey Diet (Allied Mills Inc., Chicago, Ill.) and bananas with a gradual reduction of bananas concomitant with the introduction of solid diet pellets. All cages had external water bottles with fresh water present at all times.

In the last year of this study a community cage was constructed to hold animals not in an acute state of study. This cage was 3.3 meters long, 2.5 meters high and 1.3 meters wide with sides and top of hardware cloth. Multiple feeding and watering stations were provided. This cage had numerous perchs and allowed greater mobility and social interaction. As many as 50 monkeys could be maintained in a cage of this size without overcrowding (0.15 m^3/animal). A gutter system allowed daily washing of the entire cage.

Induction of Ovulation

Ovulation was induced using the procedure described by Dukelow (1970). This system consisted of 5 days pre-treatment with progesterone dissolved in corn oil and injected daily intramuscularly (5 mg/0.25 ml), followed by 4 days of follicle stimulating hormone (FSH) (FSH-P, Armour Baldwin Laboratories, Omaha, Nebraska), injected subcutaneously (1 mg/0.20 ml). A final injection of human chorionic gonadotropins (HCG) (A.P.L. Chorionic Gonadotropin, Ayerst Laboratories Inc., New York) was administered intra-muscularly (500 i.u./0.25 ml) approximately 8 hrs after the fourth injection of FSH. Ovulation was expected 6 to 14 hrs after the HCG injection. In preliminary studies ovulation was ascertained by observation of the ovaries at laparotomy. The development of laparoscopic techniques allowed for the detection of ovulation by laparoscopic examination. The

progesterone pretreatment was eliminated in some studies to determine the effect on the response.

Laparoscopic Examination of the Ovaries

The monkeys were anesthesized by a single intraperitoneal injection of sodium pentobarbital (Halatal solution, 64.8 mg per ml, Jensen-Salsberry Laboratories Division, Richardson-Merrell Inc., Kansas City, Mo.), 0.25 ml per animal. When unconscious the animal was prepared by clipping the hair from the abdominal region and wiping the surface with zephiran chloride (Winthrop Laboratories, Division of Sterling Drug Inc., New York, 1:750 aqueous dilution). The prepared monkeys were placed in a supine position on an examination table tilted at 30°, head down in a modified Trendelenburg's position. Anesthetic hypothermia was countered with a towel covered heating pad under the animal. The hind legs were secured to the table by adjustable straps.

A midline incision, one centimeter in length, was made through the skin in the area of the umbilicus. A trocar-cannula was then inserted through the incision and the linea alba towards the posterior at approximately a 30° angle to the body. Upon entering the peritoneal cavity the trocar was removed and the laparoscope inserted through the cannula. The abdomen was insufflated with humid 5% CO_2/air as the laparoscope was being inserted. An ancillary Verres

cannula was inserted through a small incision approximately 2.5 cm posterior and lateral to the first incision. This small probe (2 mm in diameter) was used to manipulate the internal organs for better viewing and photography.

After completing the examination the main incision was closed by suturing first the muscle layer and then the skin with chromic 3-0 suture. In the first one hundred examinations no closure was made. One case of herniation occurred and after that time all monkeys received some closure. If animals were to be laparoscoped the following day only the skin was sutured. In all instances a post-examination antibiotic treatment was administered. Nitrofurazone powder (Furacin, Eaton Laboratories, Norwich Pharmacal Co., Norwich, N.Y.) was placed on the incision and then covered with nitrofurazone ointment. Each animal was injected intramuscularly with 150,000 units of Procaine Penicillin G.

The equipment used in these studies was a Wolf Model 4000 projector (150 watts) with a 135° laparoscope, 5 mm in diameter (AGA Corporation, Secaucus, N.J.). The majority of the photographic work used a Canon TL camera (Canon, U.S.A., Inc., Woodside, N.Y.) and a fast color film (High Speed Ektachrome Kodak EHB 135).

Megestrol Acetate

A contraceptive agent being clinically tested in the late 1960's and early 1970's was administered to squirrel monkeys concomitant to the ovulation induction regime. This agent was megestrol acetate (MA) (17 α-acetoxy-6-methyl-pregna-4, 6-diene-3, 20 dione (Fig. 1). It was administered either by subcutaneous injection or by silastic implants; the latter means will be discussed in the following section.

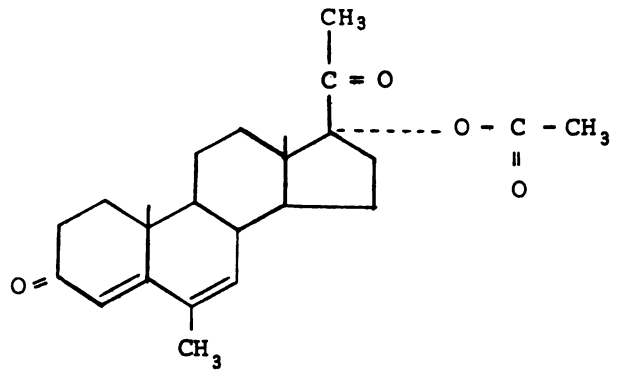

Figure 1. Structure of megestrol acetate.

The megestrol acetate (MA) for injection was dissolved in corn oil at concentrations of 200 µg, 400 µg, 1000 µg and 2000 µg per ml and injected at a dose level of 0.25 ml daily. The injections were administered on the same days as the FSH and for 1 day following the last FSH injection (a total of five daily injections). The monkeys were

subjected to laparoscopy approximately 24 hrs after the last MA injection, i.e., 36 hrs after the HCG injection.

Silastic Implants

All silastic implants were made by the author using Silastic Brand Medical-Grade Tubing (Catalog No. 602-261, 0.062 in. I.D. by 0.095 in. O.D.) and Silastic Brand Medical Adhesive (Silicone Type A, Catalog No. 891) both purchased from the Dow Corning Corporation (Medical Products Division, Midland, Mich.). Implants 30 mm in length contained a drug core 20 mm in length with a drug mass of approximately 30 mg. The distal 5 mm at each end was sealed with the medical adhesive.

Construction of Implants

The implants were constructed as follows: (1) the tubing was washed, rinsed and air dried, (2) segments 30 mm in length were cut from the washed tubing, (3) one end of each segment was sealed for a distance of about 5 mm by injecting medical adhesive into the lumen with a 5 ml plastic syringe and a disposable 22 gauge needle cut about 1 cm from the hub, (4) this semisealed implant was weighed to ± 0.1 mg on a Sartorius Selecta balance, (5) MA was added to the implant through a disposable Pasteur pipette and firmly packed using a glass rod slightly smaller in diameter than the inner diameter of the tubing, (6) the implant was

reweighed to ± 0.1 mg; this weight minus the weight in step 4 gives the mass of MA in that capsule, (7) the second end was sealed as in step 3 above and the final capsule trimmed and weighed, and (8) prior to use the implant was placed in 0.15 M NaCl for 24 hrs and then autoclaved.

In Vitro Studies

Silastic implants containing either ^{3}H-labelled or unlabelled MA were incubated in a tissue culture medium (TC-199-IX, unmodified with Earle's Salts, Catalog No. 115EE, Grand Island Biological Co., Grand Island, N.Y.) to determine the *in vitro* release rate. Each implant was placed in 10 ml of medium and sealed in a 12 ml capacity bottle. The bottles were incubated at 37°C in a humid atmosphere of 5% CO_2, 95% air. At the end of 24 hrs incubation 1 ml aliquots were removed from the bottles and 1 ml of fresh medium was added. The aliquots from the bottles containing ^{3}H-MA were transferred to liquid scintillation vials and 10 ml of Bray's solution added. The activity was determined in a Nuclear Chicago Liquid Scintillation Spectrophotometer Model Unilux III. Quenching was corrected using internal spiking with $^{3}H_2O$. Aliquots from the non-labelled implants were discarded.

The removal and addition of 1 ml of medium daily continued for 7 days. At that time the implants were transferred to 10 ml of fresh medium and the sampling

continued. This routine was continued for a total of 5 weeks. After the 5 weeks the implants were removed, air dried and weighed. These studies provided data on the amount of MA released daily, the effects of concentration in the medium on rate of release, and the linearity of daily release rate over a 5 week interval of time.

In Vivo Studies

In vivo studies were conducted to determine the average amount of MA released per day and to determine the effects of MA administered via silastic implants on the ability of the squirrel monkey to respond to induced ovulation. To determine the average amount of MA released per day, two implants were inserted subcutaneously in each of several squirrel monkeys, one implant per axillary region. The monkeys were anesthesized, prepared and received antibiotic treatment as described previously. The implants were inserted through an incision about 3.5 cm below the axillary region. A blunt probe was inserted under the skin upward towards the axilla. The probe was removed and the implant inserted into the resulting passage. The implants were removed, dried and weighed at various periods of time, up to 11 weeks after insertion. The amount of weight lost divided by numer of days inserted provided the average amount released per day.

Studies concerning the effectiveness of MA released from subcutaneous silastic implants were also conducted. The implants were inserted as described above. The ovulation induction regime (FSH phase) was begun either 11 days or 29 days after implant insertion. In some studies the progesterone pretreatment was eliminated. Ovulation was detected as previously described. The implants were weighed after removal and those data were used to supplement the release rate data described above.

Preliminary studies were conducted to determine a good method by which the amount of MA remaining in an implant could be determined. Implants were weighed before and after an *in vivo* trial. The difference in weight was assumed to be the amount of MA lost. These implants were then placed in chloroform and cut so that the drug core was dissolved. The amount of MA in the chloroform was then determined spectrophotometricly.

Accumulation Studies

One monkey with ^{3}H-MA implants *in situ* for 5 weeks was sacrificed and the internal organs analyzed for radioactivity. Weighed tissue samples were oxidized and $^{3}H_2O$ collected; activity was determined by scintillation counting. The analysis of these tissues was done in collaboration with members of the Pharmacology Department at Michigan State University.

EXPERIMENTAL DESIGN

Throughout these studies steps were taken to reduce biological variation. In the studies concerning the effects of megestrol acetate on the ovulatory response of *Saimiri sciureus*, control and test animals were from the same shipments and had received similar pretreatment care. All animals showed positive response to the induction regime prior to treatment.

The monkeys received in 1969 and 1971 used in the acclimiation-seasonality study were placed in two separate groups (1969 monkeys in Groups I and II, 1971 monkeys in Groups I and III). This procedure provided more data points for the different stages of acclimation. Differences between season and ovulation and the interaction between length of acclimation and incidence of ovulation were tested by the chi square test.

The laparoscopic examinations for ovulation were all done by this investigator. An examination for ovulation usually consisted of two observations, 24 hrs apart, in each monkey. This procedure allowed for detection of morphological changes associated with ovulation.

The silastic implants were made by the investigator. In each study care was taken to use implants that were similar. For each implant the mass, length and density of megestrol acetate core was determined. In the *in vivo* studies the implants were distributed so that the average mass of the two implants in each monkey were approximately equal.

Before the studies implants were weighed, autoclaved and reweighed to determine if the sterilization procedure resulted in drug loss. No detectable change in implant weight was found.

The Student's T-test was used to evaluate the differences between *in vivo* and *in vitro* release rates of MA from the silastic implants.

RESULTS

Detection of Ovulation

Detection of ovulation by the laparoscopic examination of the ovaries in *S. sciureus* was not difficult. Single blood vessels on the surface of a 1-mm follicle are easily discernible. Most *S. sciureus* have very little abdominal fat and the reproductive tract is not covered by layers of adipose tissue.

In most instances it was necessary to observe the follicle at least twice, with a 24-hr interval between observations. Some follicles developed to a preovulatory stage but did not ovulate. When ovulation occurred gross morphological changes took place within a 6-hr period. Two monkeys were continuously observed for 8 hrs, during which ovulation occurred. The preovulatory follicle appeared as a rounded dome 1.5 to 2 mm in diameter at the base and 0.7 to 1.0 mm in height. The top of the dome appeared clear and gelatinous. Within 1 hr following ovulation the clear area became opaque and then hemorrhagic. Luteinization began around the base of the clear area at the time of ovulation and this increased following ovulation. The peak of the follicular dome characteristically

flattened, due to loss of follicular contents. At 39 hrs, the luteinization was complete and all hemorrhagic appearance was gone. In 454 observations, 51.4% of the ovulations occurred on the left ovary, 48.6% on the right and 11% were double ovulations.

Natural ovulation occurred in three monkeys between 9 and 11 days after the induced ovulation. No significant morphological differences were noted between natural and induced follicles.

Induction of Ovulation

Using the progesterone-FSH-HCG regime described by Dukelow (1970) ovulation was successfully induced in *S. sciureus*. In preliminary studies ovulation occurred in 60% of the monkeys. Four of thirty (10.3%) monkeys in the seasonality study did not ovulate during the 1.5 years of study. The effects of the progesterone pretreatment will be discussed later.

Inhibition of Ovulation

Megestrol acetate injections.--The effects of the megestrol acetate subcutaneous injections on induced ovulation in *S. sciureus* are presented in Table 1. Forty-four observations were made in monkeys by laparotomy before the establishment of the laparoscopic technique. Some ova were recovered when the oviducts were flushed during the laparotomy. The results of forty-six observations made using the

laparoscopic technique paralleled the direct laparotomy observations and the data from both techniques are combined in Table 1. A daily dose of 50 µg of MA did not reduce the rate of induced ovulation when compared to controls subjected to the same ovulation induction scheme. A dose of 500 µg completely blocked ovulation while doses of 100 and 250 µg daily had dose-related, but nonlinear effects. By chi square test, a significant influence of MA on the ovulation followed the hormonal regime ($p < 0.005$).

Table 1

Effects of Megestrol Acetate Subcutaneous Injections on Induced Ovulation in *Saimiri sciureus*

Treatment[a]	Number of Monkeys	Monkeys Ovulating	
		Number	%
Control	30	18	60
M.A. 50 µg.	10	6	60
M.A. 100 µg.	20	7	35
M.A. 250 µg.	20	4	20
M.A. 500 µg.	10	0	0

[a]All injections 0.25 ml, corn oil vehicle, control received vehicle only.

Megestrol acetate-silastic implants.--When MA was administered to *S. sciureus* via subcutaneous silastic implants the ovulatory response was inversely related to the time *in situ* (Table 2). Monkeys with implants inserted 11 days prior to the initiation of the FSH injections exhibited an ovulation rate of 62.5%. In contrast, if the implants were inserted 29 days prior to FSH the ovulation rate was only 33.3%. In this study 50% of the control animals ovulated. All monkeys were subjected to laparoscopy, to observe follicular morphology, 12 and 36 hrs after the HCG injections.

Table 2

Effects of Megestrol Acetate-Silastic Implants on Induced Ovulation in *Saimiri sciureus*

Days (Insertion to FSH)	Control[a]	Treated[b]
11	40.0% (2/5)	62.5 (10/16)
29	60.0% (3/5)	33.3% (4/12)

[a]Control animals received no implants.

[b]Treated animals had one silastic implant containing megestrol acetate inserted subcutaneously into each axillary region either 11 or 29 days prior to the first FSH injection of the induction regime.

The progesterone pretreatment resulted in a decreased ovulation rate in monkeys with MA-silastic implants (Table 3). Implanted monkeys receiving the pretreatment ovulated at a level less than that seen in implanted monkeys without the pretreatment (25% vs. 60%, $0.10 > p > 0.05$). The effects of progesterone pretreatment on control monkeys will be presented in the section on seasonality.

Table 3

Effects of Progesterone Pretreatment on Induced Ovulation in *Saimiri sciureus* with Megestrol Acetate-Silastic Implants

	Monkeys Ovulating	
Treatment	Number	%
Control:		
Pretreatment, no implant	5/10	50
Implant:		
No pretreatment	12/20	60
Implant:		
With pretreatment	2/8	25

In vivo and *in vitro* release rates.--The *in vivo* release of MA from subcutaneous silastic implants was determined for 40 implants inserted in 20 monkeys. The mean daily release rate was calculated to be 52.2 ± 2.8 µg/day (mean ± s.e.). These implants were *in situ* either 34 or 77 days. No significant difference was noted between the values determined at 34 days compared to the values at 77 days. *In vitro* the mean daily release rate was found to be 12.6 ± 1.7 µg/day. These implants were incubated for 34 days. The *in vitro* release was influenced by concentration of the compound in the medium within 48 hours and the rate released was not linear over the 5-week period (Figure 2). The *in vivo* and *in vitro* mean daily release rates were significantly different ($p < 0.01$). No significant differences were noted between the gravimetric and spectrophotometric methods for determining amount of MA lost.

Tissue accomulation of megestrol acetate.--Using ^{3}H-MA-silastic implants *in situ* for 5 weeks, concentration of the progestin was highest in the oviducts and ovaries, less in the vagina, cervix and uterus and was not detectable in the pituitary (Table 4).

Seasonality of Ovulatory Response

Two monkeys died in April 1972 resulting in only four monkeys in the 1-year acclimated group (1970 monkeys) and nine monkeys in the nonacclimated group (1971 monkeys)

Figure 2. Accumulative amount of ^{3}H-megestrol acetate released from silastic implants incubated *in vitro*.

Table 4

Tissue Accumulation of ^{3}H-Megestrol Acetate from Subcutaneous Silastic Implants

Tissue[a]	Wet Weight of Tissue	^{3}H-MA/100 mg Wet Weight
	(mg)	(µg)
Oviducts	81.6	2.5
Ovaries	115.8	2.0
Vagina	453.4	0.8
Cervix	544.6	0.8
Uterus	232.0	0.7
Adrenals	172.2	0.7
Kidney	1,897.0	0.6
Liver	20,000.0	0.6
Thyroid	44.6	n.d.[b]
Pituitary	14.0	n.d.[b]

[a]Values for all paired organs, except kidney, are average.

[b]n.d. = not detectable.

after that date. The percent response for each level of acclimation relative to the month treated is presented graphically in Figure 3. The monkeys obtained in 1969 show an orderly response with minimal values in late summer (August and September) and maximal values in late winter (February and March). The 1-year acclimated monkeys demonstrated more fluctuation and had minimal and maximal responses in July and April, respectively. The greatest variability was found in the monkeys with the least period of acclimation, i.e., the 1971 monkeys. They had the lowest responses in February and October with a peak response in April and a minor peak in November.

In Figure 4, the percent of 2-year acclimated monkeys (1969) that ovulated in response to the ovulation induction regime is plotted with the mean hourly relative humidity recorded in the colony for each month of the study. The highest humidity was recorded in August and a low plateau was found during the cooler months of November through April. This is in diametrical opposition to the ovulatory response. Note that the progesterone pretreatment was eliminated during the last 6 months of this study yet the responses in 3 of the 4 months that the 2-year acclimated monkeys were induced are idential to those corresponding months in the previous year.

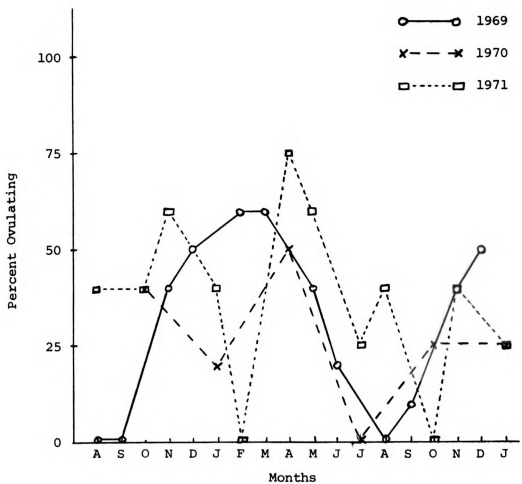

Figure 3. Ovulatory response of monkeys received in 1969, 1970 and 1971 subjected to the ovulation induction regime.

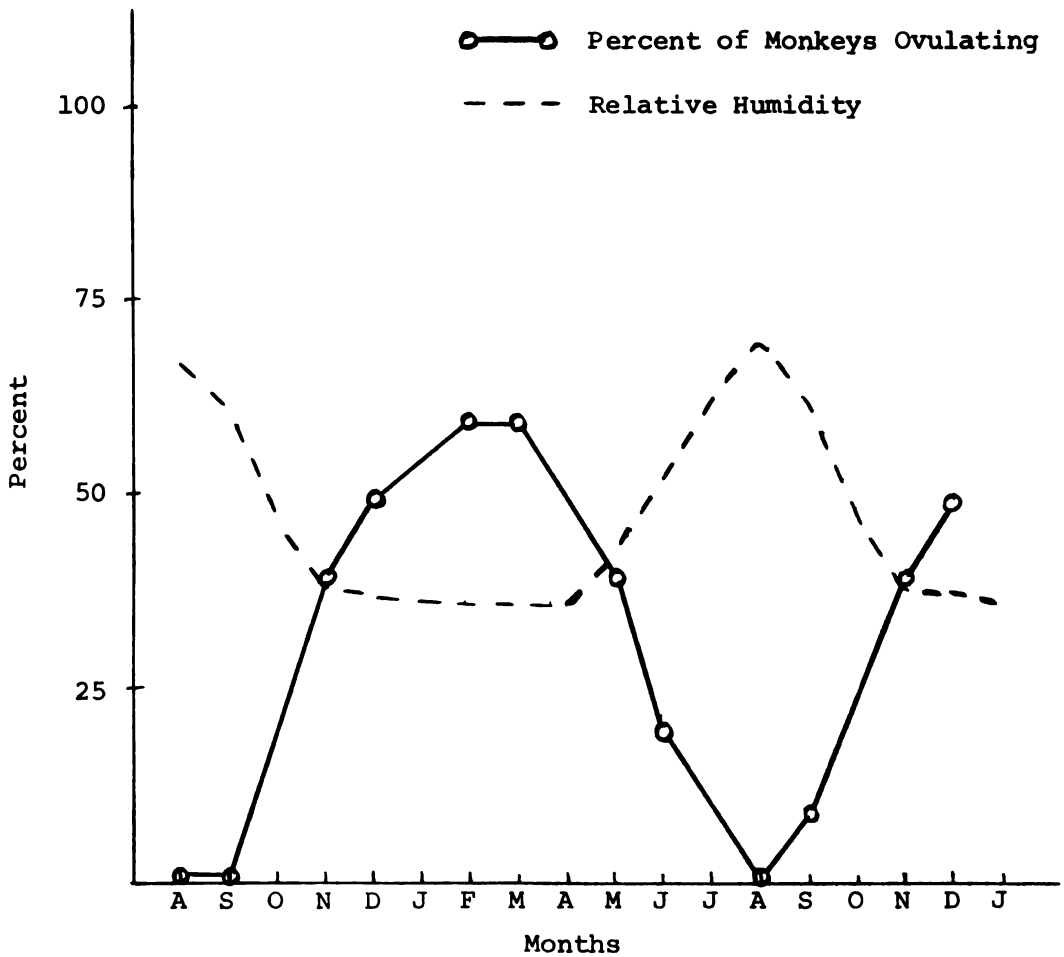

Figure 4. Ovulatory response of 2-year acclimated monkeys and the average hourly relative humidity for each month studied.

DISCUSSION

The laparoscopic examination and photography of the ovaries of *S. sciureus* is more of an art than a pure scientific task. Observation of the ovaries is not difficult. As described earlier, the lack of intestinal fat makes it easier to locate the ovaries in *S. sciureus* than in other species. It soon became clear that the bladder could be partly emptied by gentle downward expression of the abdomen while the anesthesized monkey was held in an upright position. Frequently both ovaries, oviducts and uterus could be seen clearly without use of the ancillary probe. Laparoscopic photographs were clearest when centered on a single structure with a field of view of 5 to 15 mm. Improved visualization could be achieved by bouncing the light off other tissues or by back-lighting. The laparoscope, due to the placement of light outlet and lens can be used to project light in front and behind a follicle being photographed. It is with the photography that laparoscopy becomes an art. Many hours of trial and error were necessary to find the best conditions for photographing the structures easily viewed with the eye through the laparoscope. Ektachrome EHB-135 film produced the most satisfactory results when

exposed for 1/8 second. The highest setting on the laparoscopic light projector was always used and experience provided the only gauge for proper focus.

The use of the laparoscope to view the reproductive organs in female *S. sciureus* has two main advantages over conventional laparotomy. First, morphological features on the ovaries can be seen more clearly due to the proximity of the light source and the magnifying power of the laparoscope. Single blood vessels on the surface of follicles are clearly discernible even when the follicles themselves are less than 1 mm in diameter. Second, the laparoscope allows for repeated examinations without the formation of surgical adhesions. Monkeys received in 1969 have been subjected to laparoscopy in excess of 25 times and no adhesions have resulted.

The induction of ovulation, using the method described by Dukelow (1970) does not appear to alter the physiological ovulation mechanisms. Three monkeys, received in 1969 and used in the seasonality study, were observed to have naturally ovulated between 9 and 11 days after HCG. This natural ovulation followed the induced ovulation which occurred 12 hrs after HCG. This observation would suggest that the induction regime used does not cause refractoriness to endogenous gonadotropins. These monkeys had been induced to ovulate for about 3 years at the time of this observation.

It may be assumed from the time intervals noted that the natural ovulatory cycles of *S. sciureus* maintained in a laboratory environment is about 10 days. This is in agreement with the 10-day ovulatory cycle found in *S. sciureus* by Dr. C. P. Richter (personal communication).

The antiovulatory effects of the higher concentrations of megestrol acetate injected subcutaneously were quite decisive. Follicular development was noted in some subjects while in others the ovaries appeared quiescent. The inhibition of ovulation, even when exogenous sources of FSH and LH are given, would suggest that the site of action is probably in the ovary itself. It has been suggested that, in some species, the preovulatory LH surge results in some luteinization and release of progesterone from the theca interna cells. This progesterone may act locally to change the follicular wall prior to ovulation (Baker, 1972). The high concentrations of MA accumulated in ovarian tissue in the radioactive implant study suggest that it is bound by receptors in these tissues. It is likely that the accumulation of MA on these receptors blocks the action of preovulatory progesterone on the follicle.

A second mechanism of action for MA in *S. sciureus* is suggested by the seasonality data. It would appear that the concentrations of FSH and LH administered exogenously

are not sufficient to induce ovulation unless some endogenous gonadotropins are available. Considering this, the MA probably has an antiestrogenic effect on the hypothalmic-pituitary axis to decrease the release of endogenous gonadotropins. This would also explain why the MA released from silastic implants decreased the ovulatory response more than MA administered by subcutaneous injection. The injected MA would present high blood concentrations of the progestogen initially, with a high degree of CNS gonadotropin block. The concentrations of MA would drop, depending on biological half-life of the compound, and allow some release of the gonadotropins. The silastic implants, however, maintain a relatively constant concentration of MA and therefore maintain a level of CNS blockage intermediate to the extremes seen in the injected animals.

The concentration of MA in the tissues of *S. sciureus* following 34 days of administration via subcutaneous implants agrees quite well with the results reported by Goldzieher and Kraemer (1972). In a collaborative study with investigators of the Mead Johnson Co. they found that 24 hrs after oral administration of 500 µg of radioactive megestrol acetate highest concentrations were in the liver, oviducts, ovaries and uterus. That study was conducted in baboons and the MA given in a single dose. The present study found highest concentrations in the reproductive tract

and the MA was administered continuously for 34 days. Differences in the concentration found in the livers may be due to induced hepatic microsomal enzymes. In neither study was the radioactivity characterized. The concentrations of MA in the present study were too low for extraction and characterization.

Differences between *in vivo* and *in vitro* release rates can be explained by the nature of the *in vitro* system. The compound was allowed to accumulate around the implant since the incubation was static. Rudel (1970) found that release rates from the silastic membrane into the surrounding solvent would take place only when the concentration gradient within the membrane favors outward diffusion. Dr. A. Michaels, of ALZA Research (personal communication), has stated that *in vitro* systems with a continuous flow of medium past the implants have release rates greater than those reported *in vivo*. A boundary layer forms around the implant in a static medium rapidly shifting the concentration gradient so that movement within the membrane is drastically reduced. Such continuous flow systems require extraction of the compound from large volumes of solvent. Precise determinations using a continuous flow system also require techniques and equipment not available for the present study. This study did provide some information that could not be obtained in a dynamic system, i.e., the

period of time required for the compound to reach a concentration in the medium that would decrease release rate. Another important observation derived from these studies is that *in vivo* release rates cannot be extrapolated from *in vitro* data.

The seasonality and acclimation study contribute to a better understanding of the cyclicity of *S. sciureus* and suggest why other workers have gotten a wide range of values concerning the estrous cycle in this species. Denniston (1964) found an average cycle length of 25.2 days in a study conducted over a 3-month period. In a study by Rosenblum *et al.* (1967), the most frequently observed interval between the onset of consecutive estrus periods was found to be 7 days. These monkeys were in different stages of acclimation and showed a wide variation for the first 10 months of the study. At the end of the 2-year study a shift in vaginal cornification peaks was noted so that the peak occurred early in the calendar year and not in mid-year as previously observed. Lang (1967) reported an average cycle length of 12.5 days based on vaginal smears. He found that the examination of ovaries at necropsy indicated that the majority of the estrous cycles are anovulatory. The variability of estrous cycles in these studies has been suggested to be a manifestation of environmental conditions, including size of cage (Hutchinson, 1970). A discrete seasonal cyclicity

with no shift as the study progressed was recently reported (Srivastava, Cavazos and Lucas, 1970). The lack of shift was attributed to the degree of acclimation but may have been due to the stable environmental conditions. These workers agreed that the estrous cycle of *S. sciureus* is very much subject to variation due to environmental influences. Several of the above studies were short term (less than 1 year) and the acclimation state of the animals prior to the start of the study was not stated.

The present study used monkeys at various stages of acclimation. In the last 9 months of the study the response of the animals began to show similarities between the groups held for different intervals of time. All groups at this time showed a decline in response from spring to summer with an increase in the fall. This correlates as an inverse relationship with humidity changes. A similar relationship between humidity and reproductive activity has been shown in an African monkey *(Cercopithecus mitis kolbi)* (Omar and DeVos, 1971). In the present study changes in lighting, temperature and food source were minimized. Since only relative humidity was allowed to fluctuate it is suggested that this may be the environmental stimulus that triggers reproductive activity in *S. sciureus*.

DuMond (1968) suggested a relationship between rainfall and the mating season for *S. sciureus* in the

Amazon Basin and in a seminatural environment near Miami, Florida. In the Amazon, peak rainfall occurs in mid-March with the midpoint of the mating season 5 months later. The monkeys, when acclimated to the Miami environment, have the same relationship, peak rainfall in mid-September with decreasing rainfall until late February, and a mating season midpoint 5 months after the peak rainfall. In the present Michigan study, the peak relative humidity was recorded in August and the peak ovulatory response was observed to occur 6 months later. In each location, the Amazon, Florida, and Michigan, the peak reproductive activity occurs after a decline in humidity and decreases as the humidity increases.

Changes in pheromones, temperature regulation mechanisms, and glandular secretions may be affected by changes in relative humidity and may influence the pituitary release of gonadotropins through hypothalamic neural connections. Factors other than relative humidity were considered, such as changes in husbandry and atmospheric pressure, but were subjectively eliminated as it was believed that they could not account for the uniform response seen in the 2-year acclimated monkeys. Elaborate humidity controlled studies have been considered to determine the relationship between relative humidity and ovulation but they were determined to be outside the scope of this research.

This study concerns only the gonadotropin treated ovulatory response of *Saimiri sciureus*, but it can be argued that the response to exogenous hormones would be maximal during the time when natural ovulation occurs.

SUMMARY AND CONCLUSIONS

Saimiri sciureus was studied as a model for the induction, detection and inhibition of ovulation. Megestrol acetate was administered by subcutaneous injections and silastic implants. The ovulatory response was investigated to determine differences between monkeys at various stages of acclimation and between the same monkeys at different seasons of the year. The following conclusions are indicated by the data obtained:

1. *S. sciureus* will repeatedly ovulate in response to a regime of progesterone--FSH- HCG;
2. induced ovulation can be blocked by daily injections of 500 µg megestrol acetate, reduced by dose levels of 250 and 100 µg daily and is not affected by injections of 50 µg per day;
3. the effects of megestrol acetate administered via subcutaneous silastic implants varied inversely to the length of time *in situ* and on the progesterone pretreatment;
4. the amount of megestrol acetate released from silastic implants *in vivo* is approximately 4.7 times greater than the amount released in a static *in vitro* system;

5. *S. sciureus* requires at least 18 months to acclimate to a stable laboratory environment;
6. acclimated *S. sciureus*, in a Michigan laboratory environment, in which relative humidity was allowed to fluctuate, show maximal ovulatory response in the winter and minimal response in the summer months.
7. *S. sciureus* is a good model for the laparoscopic examination of ovarian morphology and ovulation; its use in reproductive studies is limited by its seasonal responses and is enhanced by its ease of handling, adaptability and low susceptibility to surgical stress.

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APPENDICES

APPENDIX A

PRE- AND POSTNATAL CARE OF *SAIMIRI SCIUREUS*

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PRE- AND POSTNATAL CARE OF *SAIMIRI SCIUREUS*

One female *S. sciureus* received in our laboratory on October 21, 1971 was diagnosed pregnant on December 28, 1971. The following records describe her care from time of arrival until birth of her infant:

October 21, 1971--received by air shipment from Tarpon Zoo, Inc.

- injected with 150,000 units procaine penicillin G
- placed, with 4 other females from same shipment, into stainless steel cage, 66 cm deep X 80 cm high X 57 cm wide
- diet of Wayne Monkey Diet, crushed with bananas, water ad lib.
- weight 659 gms

October 28, 1971

- weight 720 gms

November 4, 1971

- weight 741 gms
- diet changed to whole biscuits and water, no fruit

November 20, 1971

- weight 849 gms

December 28, 1971

- weight 920 gms
- isolated from group, new cage 66 cm X 73 cm X 46 cm
- diet included sweetened condensed milk 3X/week

- [Note: fetus is carried high, in upper two quadrants of abdomen, labial folds swollen and very fluidy appearing.]

December 30, 1971

- eating very poorly, smaller friendly companion placed in her cage

January 3, 1972

- fetus carried lower, active and restless

January 4, 1972

- very friendly, will take apple and bananas from my hand

January 5, 1972

- blood in cage and around vulva, still has fetus

January 6, 1972

- baby on mother's back!

The infant was named Sam. Assuming a gestation length of 165 days he was conceived about August 24, 1971. Sam generally held to his mother's back, even to nurse. She was seen to hold him only twice, at about 4 days of age. At one week his eyes were open. By 18 days he moved about on his mother. Her vulva was of normal size. Sam began to vocalize at about 4 weeks of age. At 5 weeks he was riding on the back of the companion female and could grasp the cage.

April 15th, Sam was observed off the other monkeys, moved about the cage freely and appeared to taste the feed biscuits.

May 19th, Sam and the two females were placed in the community cage. At 9 months of age Sam weighed 460 gms. At the time of this writing he is 15 months old and appears very healthy.

APPENDIX B

CONSTRUCTION OF COMMUNITY CAGE

APPENDIX B

CONSTRUCTION OF COMMUNITY CAGE

A community cage was designed and constructed in April 1972. This cage has vertical corner posts and diagonal supporting posts of 2 X 4 lumber. Horizontal top and bottom pieces are of channel iron and the sides, top and ends are covered with 1/2 inch mesh hardware cloth. A door at one end is 12 inches wide, made of solid plywood. The cage is 4 ft wide, 10 ft long and 7 ft high. The cage sits on a 3/4 inch exterior grade plywood floor elevated on 8 concrete blocks. A galvanized steel gutter goes around three sides of this floor. The cage is positioned so that the entire structure can be hose washed daily.

The cage is constructed so that no supportive members of the cage are inside (all wood covered by the hardware cloth, except floor and perchs). There are 280 cubic feet of space and 31 linear feet of rounded perchs in the cage. This room would allow 60 monkeys to sit on the perchs and allows 4 cubic feet of space per monkey (minimum N.I.H. requirement is 2.7 cubic feet).

The economic value of this cage is worth noting. Thirty-five monkeys held in regular cages cost $10.50 daily maintenance cost. In the community this cost is less than $2.00 per day, a saving of more than $3,000 per year. The animals appear to enjoy the room and are more active. Fewer problems with tail sores have been noted.

APPENDIX C

ORTHOPEDIC CARE AND MEDICAL OBSERVATIONS

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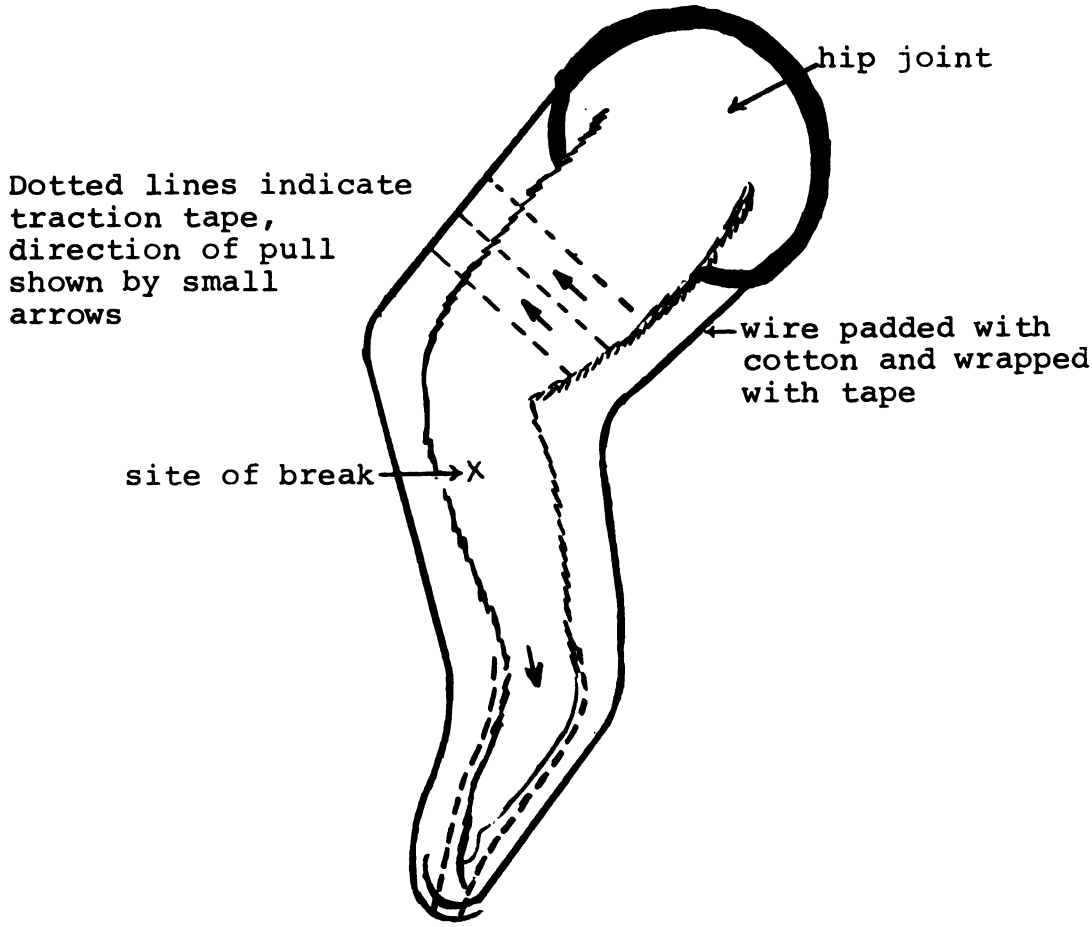
ORTHOPEDIC CARE AND MEDICAL OBSERVATIONS

A few observations concerning medical-surgical conditions in *S. sciureus* have been noted over the three years of care. These observations are recorded here to serve as references for individuals working with this species who might encounter similar situations.

The left hind leg of one monkey was accidentally broken by a student learning proper care and handling. The fibula was cleanly broken about one-third of the way between knee and ankle. A wire splint was put on the leg as diagrammed below. Pressure above the knee pulled the leg up and ventrally, traction on the foot pulled the leg distal to the break down. The entire leg was wrapped with tape around the wire. The splint was removed after 4 weeks. The muscle mass had atrophied and the monkey did not use her leg for one week. At this time she was placed in the community cage. Within three days she was using the leg well, in two months only a small lump could be felt and she was indistinguishable from the other monkeys in the cage on the basis of locomotion.


hip joint
Dotted lines indicate traction tape, direction of pull shown by small arrows
wire padded with cotton and wrapped with tape
site of break
X

APPENDIX D

PUBLICATIONS BY THE AUTHOR

APPENDIX D

PUBLICATIONS BY THE AUTHOR

Full Papers

- Megestrol Acetate: Its Effects on the Inhibition of Ovulation in Squirrel Monkeys *(Saimiri sciureus)*, by Richard M. Harrison and W. Richard Dukelow. *Journal of Reproduction and Fertility*, 25:99-101, 1971.

- Laparoscopic Examination of the Ovaries in Goats and Primates, by W. R. Dukelow, S. J. Jarosz, D. A. Jewett, and R. M. Harrison. *Laboratory Animal Science*, 21:594-597, 1971.

- Follicular Morphology and Ovulation in the Nonhuman Primate, by W. R. Dukelow, R. M. Harrison, D. A. Jewett, and M. P. Johnson. *Proceeding of the VII World Congress on Fertility and Sterility*, 1971.

- Environmental Influences in Fertilization *In Vitro* of Periovarian and Oviductal Ova, by R. M. Harrison and W. R. Dukelow. *Journal of Reproduction and Fertility*, 31:483-486, 1972.

- Natural and Artificial Control of Ovulation in Nonhuman Primates, by W. R. Dukelow, R. M. Harrison, J. M. R. Rawson, and M. P. Johnson. *Medical Primatology*, 1972.

- Factors Influencing *In Vitro* Fertilization of Periovarian and Oviductal Ova, by R. M. Harrison and W. R. Dukelow. *Proceedings of the 7th International Congress for Animal Reproduction and Artificial Insemination*, 1:525-527, 1972.

- Seasonal Adaptation of Laboratory Maintained Squirrel Monkeys *(Saimiri Sciureus)*, by R. M. Harrison and W. R. Dukelow. *Journal of Medical Primatology*, 1973.

- Megestrol Acetate: II. Effects on Ovulation in Nonhuman Primates as Determined by Laparoscopy, by R. M. Harrison, J. M. R. Rawson, and W. R. Dukelow. *Fertility and Sterility*, 1973.

Abstracts

- See the following pages for texts of abstracts.

Fed. Proc. 29:643A, 1970

INDUCED OVULATION IN THE NONHUMAN PRIMATE[1]

R. M. Harrison and W. Richard Dukelow[2]

Ovulation induction at a prescribed time was studied in squirrel monkeys. Ovulation was induced by IV or IM injection of HCG after progesterone pretreatment and various FSH sources. Superovulation was induced by the method of Bennett (*J. Reprod. Fertil.* 13:357, 1967). Forty-three percent of the animals ovulated within 12 hr post-HCG, 4 with double ovulations and the rest single. Superovulated animals averaged 4.8 ovulations. FSH was superior to PMS or HMG in promoting follicular development. Twice as many animals ovulated with 500 i.u. HCG as with 250 i.u. No difference was noted between IM or IV administration of HCG. Ovulation occurred later but with less variation in IM injected animals. Subcut. injection of 50, 100, or 250 µg of megestrol acetate (Mead Johnson & Co.) daily concomitant with the FSH resulted in 71.4, 37.5 and 20.0% ovulation when examined 40 hr post-HCG. Preliminary studies in galagos using the above procedure resulted in ovulation but it occurred later than in squirrel monkeys.

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[1]Presented at the Fed. of Amer. Soc. for Exp. Biol. Meeting, Atlantic City, N.J., April 12-17, 1970.

[2]Endocrine Research Unit (Ctr. Lab. Animal Res., Depts. An. Hus. & Physiol.), Michigan State University, East Lansing, Michigan.

LAPAROSCOPIC EXAMINATION OF OVULATION[1]

W. Richard Dukelow, S. J. Jarosz, D. A. Jewett and R. M. Harrison[2]

Determining the exact time of ovulation in domestic and laboratory animals has long been a problem in reproductive studies. Recently we have been using the laparoscope with good success to overcome this problem. Using the 135° pediatric laparoscope we have successfully examined the ovaries of rabbits, rats, pygmy goats, Toggenburg goats, guinea pigs, galagos, squirrel monkeys and Java monkeys. Observations have been made as frequently as every 4 hr for 32 hr in pygmy goats without excessive irritation or peritonitis. Light tranquilization and a local anesthetic are required. A second entry tactile probe allows manipulation of the organs for observation. Distention of the abdominal cavity with CO_2/air is essential for good observation. Using this technique it has been possible to time ovulation within 1 hr in over 60% of our monkeys and goats examined. The laparoscope offers an excellent tool for examination of internal physiology function with minor disturbance to the animal.

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[1]Presented at the 21st Ann. Session Amer. Assoc. for Lab. Anim. Sci., Chicago, Ill., November 2-6, 1970.

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Fed. Proc. 30:595A, 1971

OVIDUCTAL-UTERINE RELATIONSHIPS TO SPERM CAPACITATION[1]

R. M. Harrison, M. P. Johnson and W. R. Dukelow[2]

An *in vitro* system for the fertilization of rabbit ova was used to study the influence of the oviduct on fertilization. Ova recovered from superovulated does were incubated with sperm from the uterine horns of mated does. The incubation medium was modified acidic saline containing 20% heated rabbit serum. Gametes were incubated at 38°C for 24 hr under a humid atmosphere of 5% CO_2/air. Ova recovered from the ovarian surface 11.75 hr after HCG fertilized at a rate of 56% compared to 50% for ova recovered from the oviduct. Uterine sperm recovered 17 hr after mating fertilized 37% of the ova compared to 30% for sperm recovered 11 hr after mating. Uterine sperm recovered 11 hr after mating from a ligated uterine horn fertilized 24% of the ova compared to 35% using nonligated uterine sperm. With *in vivo* studies, 8 and 10 hr sperm from ligated uterine horns fertilized 67 and 70% of 4 hr old ova, respectively.

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[1]Presented at the Fed. of Amer. Soc. for Exp. Biol. Meeting, Chicago, Ill., April 16, 1971.

[2]Endocrine Research Unit (Ctr. Lab. Animal Res., Depts. An. Hus. & Physiol.), Michigan State University, East Lansing, Michigan.

These studies suggest that exposure to the oviduct makes the ova more susceptible to changes in environmental conditions but that the oviduct is not essential for capacitation to occur in 11 hr or less.

FOLLICULAR MORPHOLOGY NEAR OVULATION IN MACACA FASCICULARIS[1]

D. A. Jewett, R. M. Harrison, M. P. Johnson and W. R. Dukelow[2]

In the course of 189 laparoscopic examination of 31 female Cynomologous Macaques *(Macaca fascicularis)*, ovarian morphology was observed and photographed for the purpose of determining the characteristics of pre- and post-ovulatory follicular development. Serial observations were begun on day 10 of the menstrual cycle and continued for 6 days at intervals varying from 6 to 24 hours. Generalized swelling and darkening of the ovary at the site of the developing follicle could not be identified until 20 to 24 hours prior to ovulation. The presence of a relatively large, coiled vessel near the follicular cone, smaller vessels around the base, and a single small vessel transecting the follicular membrane were found to be the most reliable indications of immediate preovulatory development, and were not found earlier than 8 hours prior to ovulation. The most significant post-ovulatory morphological changes were the

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[1]Presented at the Society for the Study of Reproduction, Boston, Mass., June 30, 1971.

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diffusion of the small vessels at the base of the follicular cone, the occlusion of clear areas in the follicular membrane, and flattening or irregularity in the follicular dome. These changes were clearly evident 10 to 24 hours following oculation; and, therefore, the corpus hemorrhagicum can be easily distinguished morphologically from the pre-ovulatory follicle. This investigation demonstrated that the time requirement for the formation of the follicular dome and related vasculature is not more than 20 hours; that ovulation can be diagnosed by laparoscopy; and that comparative follicular morphology during the late stages of development will permit future accurate prediction of ovulation time by a single laparoscopic examination.

FOLLICULAR MORPHOLOGY AND OVULATION INDUCTION IN THE NONHUMAN PRIMATE[1]

W. R. Dukelow, R. M. Harrison and D. A. Jewett[2]

Thirty-one female *Macaca fascicularis* were examined by laparoscopy to evaluate ovarian morphology and follicular development near ovulation. Generalized swelling and darkening of the ovary characterized the developing follicle 24 hr prior to ovulation; a specific pattern of blood vessels associated with the follicle developed 8 hr prior to ovulation. Deterioration of vasculature and occlusion of previously clear areas on the follicular membrane were evident 24 hr after ovulation. Based on these examinations precise mating trials were carried out. Four pregnancies were obtained, and two were allowed to go to term with gestation lengths of 164 days, 15.5 hr and 165 days, 10.8 hr.

Laparoscopy was also used to ascertain the efficacy of a *Saimiri sciureus* ovulation induction scheme consisting of 5 days of progesterone (5 mg); 4 days of FSH (1 mg); and a final injection of 500 i.u. HCG. This scheme was used to study the effectiveness of various contraceptive agents in

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[1]Submitted to 7th World Cong. on Fert. and Steril., Tokyo and Kyoto, Japan, October 17-25, 1971.

[2]Endocrine Research Unit (Ctr. Lab. Animal Res., Depts. An. Hus. & Physiol.), Michigan State University, East Lansing, Michigan.

blocking induced ovulation. Such ovulation was completely blocked with 500 mg megestrol acetate. The ovulation scheme was also effective in *Galago crassidaudatus*.

FACTORS INFLUENCING *IN VITRO* FERTILIZATION OF PERIOVARIAN AND OVIDUCTAL OVA[1]

Richard M. Harrison and W. Richard Dukelow[2]

In vitro fertilization provided a means to study the fertilizability of rabbit ova and the capacitation of sperm. Although no significant difference was observed between periovarian and oviductal ova under optimal conditions a significant difference was observed favoring periovarian ova when conditions were unfavorable. Uterine sperm recovered 17 hrs after mating have a higher fertilization rate than 11 hr sperm. Sperm recovered from a uterus with a patent uterotubal junction fertilized ova at a higher level than those recovered when the junction was ligated, although capacitation had occurred in both conditions in less than 11 hours. The data indicates that exposure to the oviductal environment influences the fertilizability of ova but is not required for sperm capacitation to occur.

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[1]In the Proceedings of the 7th International Cong. Animal Reprod. and Artificial Insemin., Munich, Germany, 1972.

[2]Endocrine Research Unit (Ctr. Lab. Animal Res., Depts. An. Hus. & Physiol.), Michigan State University, East Lansing, Michigan.

Fed. Proc. 31:278, 1972

STUDIES OF OVIDUCTAL FLUID AND *IN VITRO* FERTILIZATION IN RABBITS AND NONHUMAN PRIMATES[1]

M. P. Johnson, R. M. Harrison and W. R. Dukelow[2]

Rabbit oviductal fluid (OF) was collected with an intraabdominal flask, centrifuged, and frozen until use. Some oviductal fluid was fractionated on Sephadex G-25 and G-75. Capacitated sperm were recovered 17 hr. after mating. The sperm were incubated in 50% OF in saline with 20% rabbit serum for 1 hr. Control sperm were incubated in saline. Ova were recovered 12.5 hr after HCG and added to the sperm suspensions; then, examined after 24 hr for cleavage. OF treated sperm caused cleavage in 25% of the ova compared to 53% with controls. These studies suggest the presence of a factor in the OF which effects the fertilizing ability of sperm. Studies on the *in vitro* fertilization of squirrel monkey ova were similar except that we used either 20% heated (55°C for 20 min) squirrel monkey serum or agamma fetal calf serum in TC-199. In addition, 25 μg/ml estrone sulfate was added. These media supported sperm motility

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[1]Presented at the Fed. of Amer. Soc. for Exp. Biol. Meeting, Atlantic City, N.J., April, 1972.

[2]Endocrine Research Unit (Ctr. Lab. Animal Res., Depts. An. Hus. & Physiol.), Michigan State University, East Lansing, Michigan.

and attachment to the zona pellucida. (Supported by NIH Contract No. 70-2061, USPHS Grant No. 5-PO6-RR00366-04, to the Center for Laboratory Animal Resources, and NIH-CDA 1-K4-HD35, 306-01.)

FACTORS INFLUENCING OVULATION IN SQUIRREL MONKEYS *(Saimiri sciureus)* MAINTAINED IN A LABORATOY ENVIRONMENT[1]

Richard M. Harrison and W. Richard Dukelow[2]

Adult female squirrel monkeys (Brazilian type) were housed in double unit modular cages in an environment with controlled 12 hr light-dark cycles and a constant temperature of 22°C ± 1°C. All monkeys were supplied by Tarpon Zoo, Inc. over a two year period. Monitoring of temperature and relative humidity was continuous. Studies were conducted to determine ovulatory response to a hormonal regime of progesterone-FSH-HCG (*J. Reprod. Fertil.* 22:303, 1970). Routes of administration and dose levels of a progestational compound, megestrol acetate (MA) used to block ovulation were determined. Ovulation was detected laparoscopically 12 to 36 hr after HCG (*J. Reprod. Fertil.* 25:99, 1971).

Our studies indicate that there are seasonal responses to the induction regime related to the same factors that influence seasonal mating in the wild and in seminatural environments (DuMond, Ch. 4, *The Squirrel*

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[1]Presented in the *Proceedings* at the 4th International Congress of Primatology, Portland, Oregon, August 15-18, 1972.

[2]Endocrine Research Unit (Ctr. Lab. Animal Res., Depts. An. Hus. & Physiol.), Michigan State University, East Lansing, Michigan.

Monkey, Ed. Rosenblum and Cooper, Academic Press). Monkeys acclimated to our environment for 2 years did not respond to induction during September (relative humidity 70%) but 50% did respond during December (relative humidity 35%). During periods of maximal response ovulation could be blocked with subcutaneous injection of MA (500 µg/day) or with silastic implants containing MA. The daily rate of release of MA from the implants is approximately 50 µg, a level ineffective when injected.

These studies indicate that the relationship between environmental factors and ovulatory response has important implications in the use of *S. sciureus* for contraceptive evaluation of compounds. (Supported by NIH Contract 70-2061, USPHS Grant 5-PO6-RR00366-04 to the Center for Laboratory Animal Resources, NIH Research Career Development Award 1-KD4-HD35306-01, and Mead Johnson Research Center.)

ENVIRONMENTAL INFLUENCES ON OVULATION IN CAPTIVE SQUIRREL MONKEYS (*Saimiri sciureus*)[1]

Richard M. Harrison and W. Richard Dukelow[2]

Saimiri sciureus is one of the most popular primates for biological research. We studied seasonal variations in the response to induced ovulation. The monkeys were maintained in an indoor colony at 22 ± 2°C (12 hr light cycle). Three groups were induced to ovulate by a regime of progesterone, FSH and HCG (*J. Reprod. Fertil.*, 22:303, 1970). Each group was induced 4 times a year. The relative humidity was recorded on a continuous basis. Monkeys acclimated to our colony for 21 months prior to the start of the study did not ovulate (0/15) in Aug.-Sept. (rel. humid. 70%), in Nov.-Dec. 7/15 ovulated and in Feb.-Mar. 8/15 ovulated, in those months rel. humid. averaged 33 to 37%. A group received immediately prior to the study responded at levels of 40%, 60%, 0% and 60% ovulation in the months of Aug., Nov., Feb. and May, respectively. The results indicate that *Saimiri* maintained in a laboratory

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[1]Presented at 23rd Annual Mtg. Amer. Assoc. for Lab. Anim. Sci., St. Louis, Mo., October 15-20, 1972.

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show seasonal responses to exogenous hormones. Shifts in response peaks are related to the degree of acclimation and the environmental stimulus appears to be the level of relative humidity. Supported by NIH Career Development Award No. 1-K4-HD35, 306-01; and USPHS Grant No. 5-PO6-PR 00366-04 to the Center of Lab. Animal Resources.

Fed. Proc. 32:283, 1973

CONTROL OF OVULATION IN SQUIRREL MONKEYS[1]

R. M. Harrison, D. E. Wildt and W. R. Dukelow[2]

Ovulation induced in squirrel monkeys with progesterone-FSH-HCG can be inhibited by megestrol acetate (MA) injections. We studied MA administration by silastic implants, disposition *in vivo* and rate of release. The effectiveness of MA implants depends on induction pretreatment and time *in situ*. Monkeys receiving progesterone pretreatment ovulated at a lower rate (2/8; 25%) than those not receiving progesterone (12/20; 60%). Monkeys with implants 11 days prior to FSH ovulated at a rate of 62% (10/16) compared to 33% (4/12) for those with implants 29 days. Using MA-H^3 implants, concentration of the progestin was highest in ovaries and oviducts, less in adrenals, uterus and vagina, and not found in the pituitary. *In vivo* release rate was determined with 40 implants in 20 monkeys. To determine *in vitro* release rate each implant was incubated in 10 ml of TC-199 at 37°C for 5 weeks. One ml aliquots were removed and replaced daily and implants

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[1]Presented at the Fed. of Amer. Soc. for Exp. Biol. Meeting, Atlantic City, N.J., April 18, 1973.

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were transferred each week to fresh medium. The *in vivo* release rate was 56 ± 12 µg/day, whereas the *in vitro* rate was 13 ± 5 µg/day: *In vitro* release was influenced by build-up in the medium within 48 hours, and the rate was not linear over the five week period.

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