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REPRODUCTIVE PHYSIOLOGY OF MACACA FASCICULARIS
1. SEASONAL EFFECTS IN THE MALE
2. SEMEN PRESERVATION
3. LAPAROSCOPY

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James P. Mahone

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REPRODUCTIVE PHYSIOLOGY OF *MACACA FASCICULARIS*

1) SEASONAL EFFECTS IN THE MALE

2) SEMEN PRESERVATION

3) LAPAROSCOPY

By

James Patrick Mahone

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ABSTRACT

REPRODUCTIVE PHYSIOLOGY OF MACACA FASCICULARIS
1) SEASONAL EFFECTS IN THE MALE
2) SEMEN PRESERVATION
3) LAPAROSCOPY

By

James Patrick Mahone

These studies were conducted to determine the effect of season on several reproductively important variables in laboratory housed male cynomologus macaques. Additionally, semen collection and preservation were investigated in the male while the effect of extensive laparoscopy on reproductive performance was examined in female M. fascicularis.

Seasonal reproduction in laboratory housed adult male M. fascicularis was evaluated through examination of changes in body weight, sperm concentration and testis volume over a 13 lunar month period. No effect ($p > 0.05$) of season on body weight or sperm concentration was observed. Testis volume was significantly greater ($p < 0.05$) during the months of July through early September than during October through January (the times of greatest difference in mean testicular volumes) as well as during a standardized comparison interval with mean values during May through July being greater than during October through January.

Semen was collected from males using a rectal probe for electroejaculation. Power was supplied to the probe by a square wave generator. Pulse width ranged from 1.8 to 7.0 msec, pulse frequency from 35 to 75 Hz with a 40 volt maximum output.

The effect of varying semen extender components and the equilibration times of spermatozoa with glyceral prior to pellet freezing was examined. No significant difference was observed between motilities at extender egg yolk concentration of from 10 to 40%. Progressive motility was significantly greater at pH's of 7.2 and 8.0 than at 5.8, 6.5 or 8.7 ($p < 0.05$). Glycerol concentrations of 7 and 10% yielded optimum motility after freezing. A one minute equilibration of extender containing glycerol resulted in greater motility after freezing than did equilibration with glycerol for 25 or 45 minutes ($p < 0.05$).

Thirty-six mating trials were conducted over an eleven month period with sixteen female cynomologus macaques that had been extensively laparoscoped. Five (31.3%) of these females conceived. Two abortions from unknown causes occurred at 112 and 123 days gestation to animals which had been laparoscoped 43 and 54 times previously. Three females that had been laparoscoped 38, 47 and 49 times prior to breeding gave birth to live healthy infants.

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INTRODUCTION

Nonhuman primates are used extensively in biological research providing the closest approximation to man for many experimental investigations. Despite this need, economic factors as well as the conservation of wild primate populations place a premium on the use of these animals for research purposes. As a result, captive breeding colonies may become a vital source of supply for future research subjects.

The low fertility of captive nonhuman primates acts as a major hurdle to the development of successful breeding programs. One of the factors contributing to reduced fertility is the seasonal effect on reproduction in both sexes of many species. Previous studies have established the nonseasonality of ovulation in the laboratory housed female cynomologus macaque. Investigation was needed to determine if this same condition exists in the male of that species.

The success of artificial insemination techniques using frozen semen in domestic animals is indicative of its potential value in a primate breeding colony. Application of this technique would have the potential for obviating seasonal effects on reproduction as well as allowing

extension of the breeding potential of superior males. No live birth of a nonhuman primate from frozen semen has been reported, indicating the need for research in this area.

Knowledge of the time of ovulation is essential for any intensive breeding regimen. Laparoscopy provides the most rapid and reliable method of ovarian observation in nonhuman primates and as such is invaluable when artificial insemination or timed matings are used. The effect of repeated use of this procedure on normal reproductive cyclicity and pregnancy was unknown at the time of initiation of these studies.

The objectives of these investigations were:

1. To quantify changes in reproductive variables in the male Macaca fascicularis throughout the year.
2. To develop a method for routine semen collection in Macaca fascicularis.
3. To examine the effect of alteration of semen extender components and the effect of short equilibration periods of spermatozoa with glycerol on the motility of cynomologus macaque sperm after freezing.
4. To evaluate the effect of laparoscopy on fertility in the female Macaca fascicularis.

1) SEASONAL REPRODUCTION IN THE LABORATORY HOUSED MALE CYNOMOLOGUS MACAQUE, *MACACA FASCICULARIS*

SEASONAL REPRODUCTION IN THE LABORATORY HOUSED MALE CYNOMOLOGUS MACAQUE, *MACACA FASCICULARIS*

Abstract

Changes in body weight, sperm concentration and testicular volume were monitored for a 13 lunar month period in seven laboratory housed adult male *M. fascicularis*. No significant change was noted in body weight or sperm concentration between periods of maximal and minimal mean values nor between periods standardized for comparison between the three parameters. Testis volumes were significantly greater ($p < 0.05$) during the months of July through early September and during the standardized period of May through July than during January through March and the standardized interval October through January.

Introduction

A large number of mammals are seasonal breeders being reproductively efficient for only brief periods during the year. Among the environmental influences that may trigger this phenomenon, photoperiod, temperature and humidity are the most important.

Until recently nonhuman primates were not thought to be seasonal breeders (Zuckerman, 1931a,b). Recent evidence however, has revealed a seasonal influence in many primate species. Included in this list are ringtailed lemurs, Lemur catla (van Horn, 1975); Macaca fuscata (Namura et al., 1971); M. mulatta (Southwick et al., 1961 and 1965; Koford, 1965; Prakash, 1958, 1960); M. radiata (Simonds, 1965), Presbytis entellus (Jay, 1965); Papio hamadryas (Kummer, 1968); Cercopitheus kolbi (Omar and DeVos, 1971); M. sylvanus (MacRoberts and MacRoberts, 1966), Saimiri sciureus (Dumond, 1968; Harrison and Dukelow, 1973) and M. fascicularis (Dr. I. Berndston, 1976, personal communication).

While seasonal fluctuation in primate births have been examined in feral groups, other criteria are more discriminating determinants of the male reproductive state. Among these are gross changes in body morphology, testicular size characteristics, semen characteristics, mating behavior and hormonal influences.

Dumond and Hutchison (1967) observed increases in whole body weight and heaviness of the upper torso now termed the "fatted male" phenomenon in S. sciureus. This condition persists under constant laboratory conditions (Clewe, 1969). Androgen treatment of castrated male S. sciureus resulted in increased body size, weight and physical appearance similar to the condition found in intact animals during the mating season (Nadler and Rosenblym, 1972).

Sade (1964) reported a seasonal fluctuation in testis size in M. mulatta at Cayo Santiago, Puerto Rico. The greatest length and width were observed during the warm wet seasons during which the period of daylight was lessening. This was in contrast to decreased testicular size during the cool dry months of increasing photoperiod. The color of the male sex skin was brightest during the season of greatest testicular size and least intense during the season corresponding to diminished testicular dimensions.

Rhesus monkeys demonstrate maximum testicular tubule development during the breeding season (September through November) with all spermatogenic and spermiogenic stages present in high numbers (Conway and Sade, 1965). Degenerative changes appeared starting in December and reached a maximum from January through May. In June and July development was reinitiated and progressed rapidly.

Zamboni et al. (1974), examined semen production in male rhesus at Cayo Santiago. During the breeding season semen production was normal in terms of volume, quality and physical characteristics. Samples had a highly uniform population of motile, structurally normal sperm. During the nonbreeding season, males did not respond to electrostimulation or produced only minimal quantities of ejaculate with abnormal physical characteristics.

Drickamer (1974) theorized that the onset of the fall mating behavior in rhesus is a result of interaction of many factors. The onset of mating was observed to follow vegetation changes as a result of heavy rains in the late summer (Vandenbergh and Vessey, 1968). Gordon et al. (1976) described copulatory activity as being restricted to a five month period commencing in the fall of each year in rhesus maintained in an outdoor compound in Lawrenceville, Georgia.

The seasonal pattern of mating behavior and testosterone rhythmicity observed in the wild, is maintained for years following introduction of the male rhesus into the laboratory environment (Robinson et al., 1975).

The rhesus monkey is a widely used primate research model; however, this seasonal reproductive phenomenon is much more marked in this species than is found in humans or in some other nonhuman primate species. Research using a different, less reactive, primate would seem more appropriate.

Dang (1977) examined 275 menstrual cycles over a 3 year period in 55 female M. fascicularis. These animals were housed in a laboratory in Paris at constant humidity and temperature but under natural daylight. There was no statistical difference in conception rate between four daylight ratios examined (Short, 8-9 hours, increasing from 9-15 hours; long, 15-16 hours, decreasing from 16 to 8 hours). No seasonal difference was observed in duration of menstrual cycle length. Dukelow (1977) reported a slight seasonal effect for cycle length, with longer cycles occurring during the spring of the year and with slightly shorter cycles in the winter months. No seasonal effect was noted in the length of menstrual cycle flow. The M. fascicularis in this study were observed for a five year period. Lighting was controlled at a 12:12 hour light:dark cycle with temperature regulated between 21-26°C and relative humidity fluctuating between 40-60% according to season. Both of these investigations emphasize the difference in the seasonality of reproduction in this species compared with the rhesus female.

No information is available on the persistence of seasonal reproductive patterns in the laboratory housed male cynomologus macaque. The objective of the present study was to define the extent of seasonal reproduction in the laboratory housed male M. fascicularis. Three criteria were used to evaluate the reproductive status of the males:

1. Concentration of spermatozoa.
2. Testis volume.
3. Body weight.

Materials and Methods

This study was conducted from April, 1976 through March, 1977 with a colony of 7 adult male M. fascicularis at the Endocrine Research Unit. All animals were adults and had been in the colony for periods ranging from five months to nine years at the time this study was initiated. Five of the seven males were captive born. The macaques were individually housed in 122 cm double unit modular cages in quarters controlled at 21-26°C. Lighting was controlled on a 12:12 hour light:dark cycle (0600-1800). The relative humidity fluctuated between 40-60% according to season. Females were individually housed in the same room. The number of females present ranged from 2 to 28 over the 12 month period. All data were recorded on a lunar month (28 days) basis.

Animals were anesthetized with an intramuscular injection of Ketamine HCL (Vetalar, Parke Davis) at a dosage of 10 mg/kg body weight before sampling procedures. Every 28 days animals were anesthetized for three days in succession between 1700 and 1900 hrs. Testicular measurements and body weights were obtained during this interval. Body weights for the three day period were averaged. Three testicular

measurements were taken with a micrometer on the length, breadth and width of each testis on each of the three days. Nine values were obtained for each 3 days interval and averaged.

The testis volume was assumed to approximate that of an ellipsoid. The volume was obtained according to the formula for an ellipsoid, $V = 4/3\pi(LXWXB)$. Both calculated testis volumes were averaged, and the single value obtained was statistically analyzed.

Semen was collected by electroejaculation (described in the Materials and Methods section on Semen Collection and Preservation) using a rectal probe. After incubation at 37°C for 30 minutes, the liquid ejaculate was diluted (1:20) with distilled water to stop all sperm motility. The spermatozoa concentration was then determined with a hemocytometer. As other experiments were in progress using the male macaques, the first ejaculate taken after weighing and testis measurements was used. This allowed a minimum of one week to elapse between electroejaculation to eliminate any effect of frequency of semen collection on sperm concentration.

All data for sperm concentration, body weight and testis volume were analyzed using Scheffe's interval (Scheffe, 1953) to contrast data selected after the experiment was conducted. This procedure was used as no information is available on when seasonal peaks are to be expected in the laboratory

housed males. If drifting of seasonal reproductive peaks or re-emergence of seasonal patterns occurred more restrictive analyses would be unable to detect them.

Results

Two separate statistical comparisons were made for each factor examined (i.e., sperm concentration, mean testis volume and mean body weight).

The first comparison involved contrasting mean values for three consecutive months of highest values with the corresponding three consecutive months during which the lowest levels were observed. A second comparison was made involving a standard non-breeding season (July through the end of August) versus the pinnacle of the breeding season of feral nonhuman primates (October through January).

Sperm Concentration

The results of fluctuations in sperm concentration for all 7 males over the thirteen lunar month examination period are presented in Figure 1. The seasonal comparisons that were statistically analyzed based on this data were:

I July (6-8), Aug. (3-5), Aug. (31-Sept. 2)
vs.
Sept. (28-39(, Oct. (26-28), Nov. (23-25)

II July (6-8), Aug. (3-5), Aug. 31-Sept. 2
vs.
Oct. (26-28), Nov. (23-25), Dec. (21-23), Jan. (19-21).

Figure 1. Variations in sperm concentration over a 13 lunar month period in laboratory housed male, Macaca fascicularis.

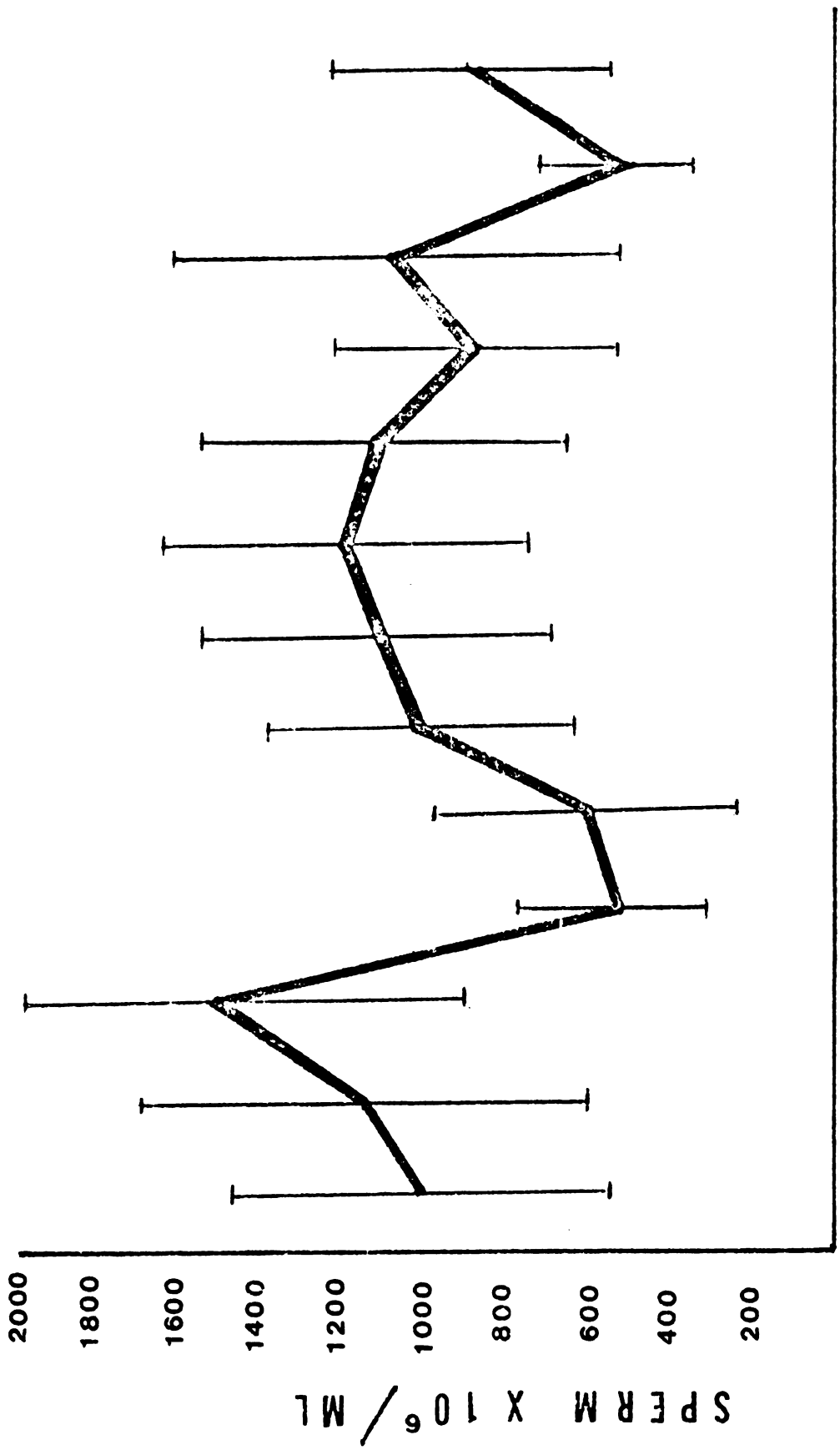

SPERM X 10⁶/ML
2000
1800
1600
1400
1200
1000
800
600
400
200
APR 13 15
MAY 11 13
JUN 8 10
JUL 6 8
AUG 3 5
AUG 31 SEP 2
SEP 28 30
OCT 26 28
NOV 23 25
DEC 21 23
JAN 19 21
FEB 15 17
MAR 15 17
LUNAR MONTHS

Figure 1

There was no significant difference ($p > 0.05$) between sperm concentrations for either of the contrasts made.

Testis Volume

Figure 2 reflects changes in testis volume over the thirteen month period. Based on the mean values for each month the following comparisons were made:

I Jul. (6-8), Aug. (3-5), Aug. 31-Sept. 2
vs.
Jan. (19-21), Feb. (15-17), Mar. (15-17)

II Jul. (6-8), Aug. (3-5), Aug. 31-Sept. 2
vs.
Oct. (26-28), Nov. (23-25), Dec. 21-23, Jan. (19-21)

Testis volume was significantly greater ($p < 0.05$) during the months of July through the beginning of September when compared to the interval January through March (representing the lowest testes volume attained. Furthermore, this same difference was observed to exist ($p < 0.05$) when a comparisons of the standard breeding seasons were made.

Body Weight

Figure 3 outlines variation in mean body weight over the study. The periods during which high and low values were compared as well as "standard" seasonal comparison were:

I May (11-13), June (8-10), July (6-8)
vs.
Oct. (26-28), Nov. (23-25), Dec. (21-23)

II July (6-8), Aug. (3-5), Aug. 31-Sept. 2
vs.
Oct. (26-28), Nov. (23-25), Dec. (21-23), Jan. (19-21).

Figure 2. Variations in average testis volume over a 13 lunar month period in laboratory housed male, Macaca fascicularis.

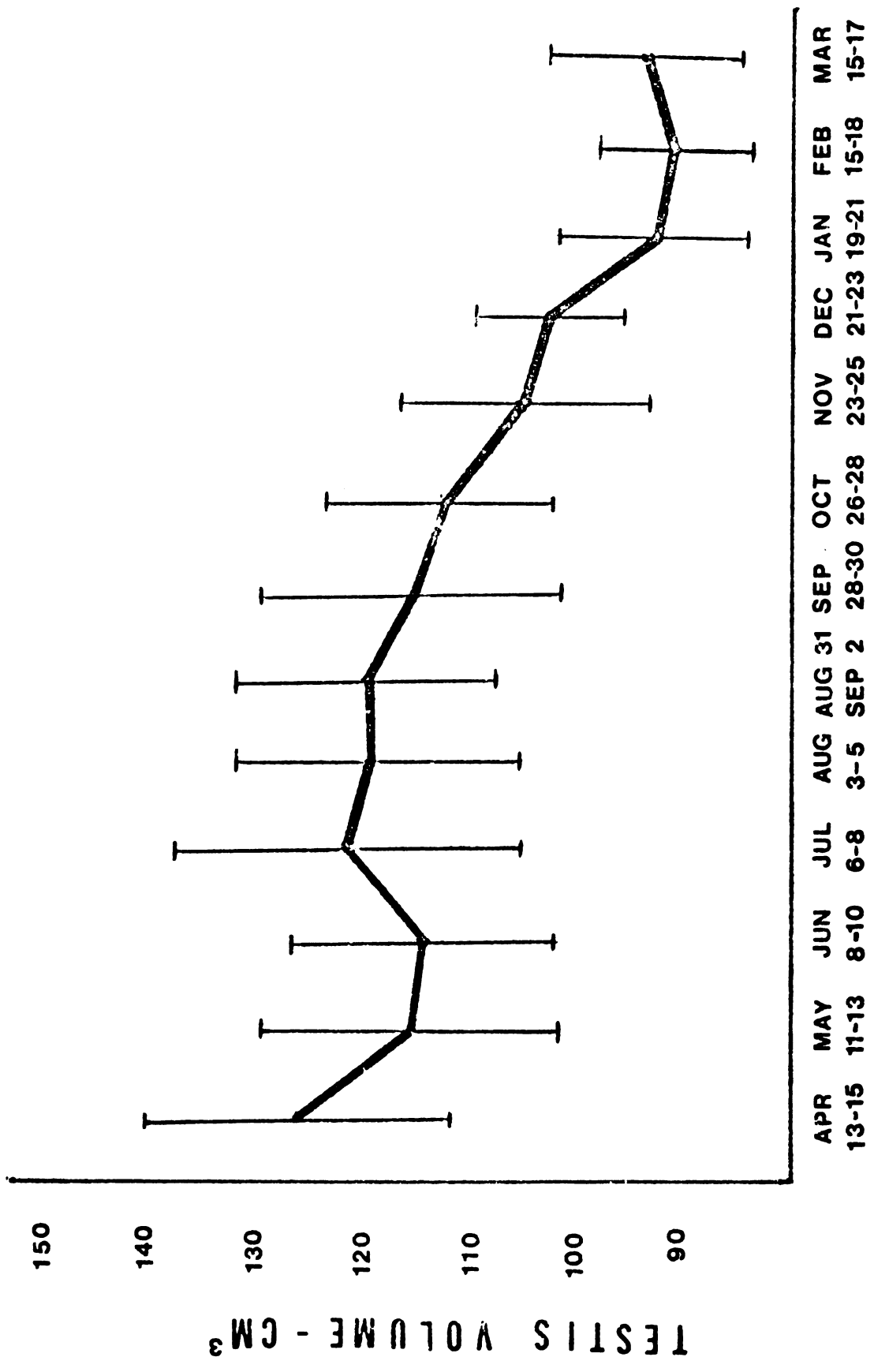

TESTIS VOLUME - CM³
150
140
130
120
110
100
90
APR 13-15
MAY 11-13
JUN 8-10
JUL 6-8
AUG 3-5
AUG 31 SEP 2
SEP 28-30
OCT 26-28
NOV 23-25
DEC 21-23
JAN 19-21
FEB 15-18
MAR 15-17
LUNAR MONTHS

Figure 2

Figure 2. Variations in average testis volume over a 13 lunar month period in laboratory housed male, Macaca fascicularis.

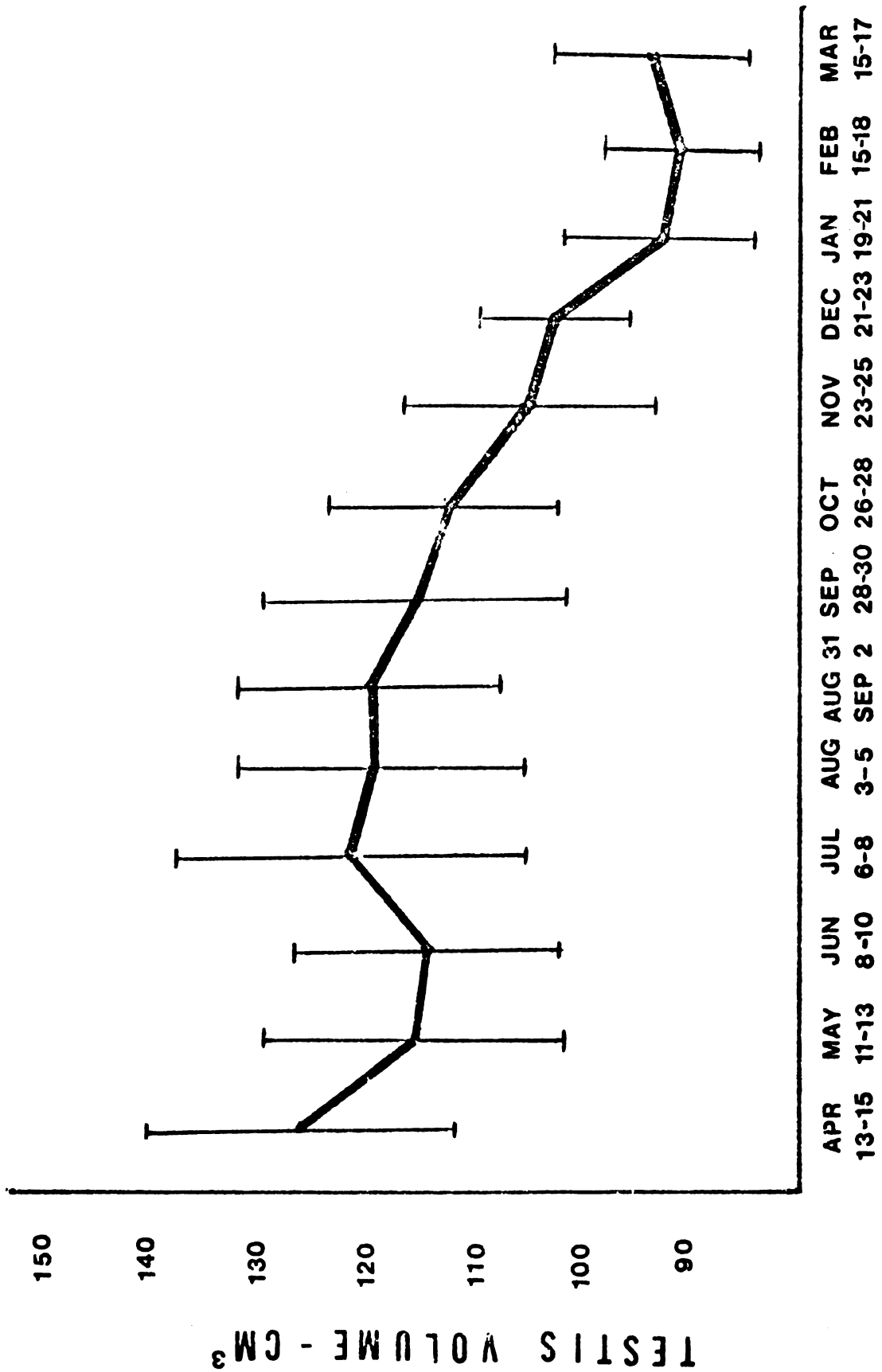

TESTIS VOLUME - CM³
150
140
130
120
110
100
90
APR 13-15
MAY 11-13
JUN 8-10
JUL 6-8
AUG 3-5
AUG 31 SEP 2
SEP 28-30
OCT 26-28
NOV 23-25
DEC 21-23
JAN 19-21
FEB 15-18
MAR 15-17
LUNAR MONTHS

Figure 2

Figure 3. Variations in body weight over a 13 lunar month period in laboratory housed male, Macaca fascicularis.

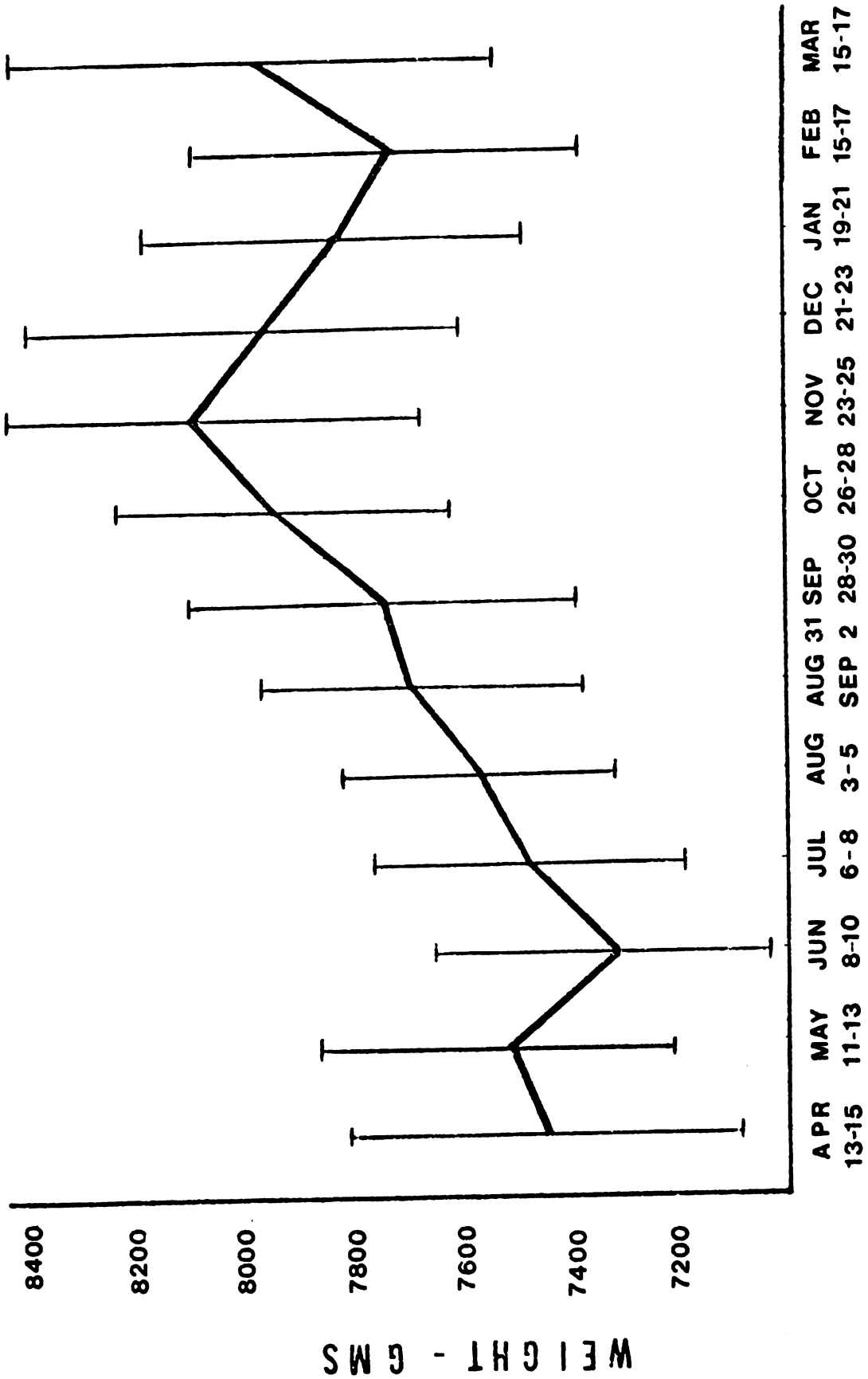

WEIGHT - GMS
8400
8200
8000
7800
7600
7400
7200
APR 13-15
MAY 11-13
JUN 8-10
JUL 6-8
AUG 3-5
AUG 31 SEP 2
SEP 28-30
OCT 26-28
NOV 23-25
DEC 21-23
JAN 19-21
FEB 15-17
MAR 15-17
LUNAR MONTHS

Figure 3

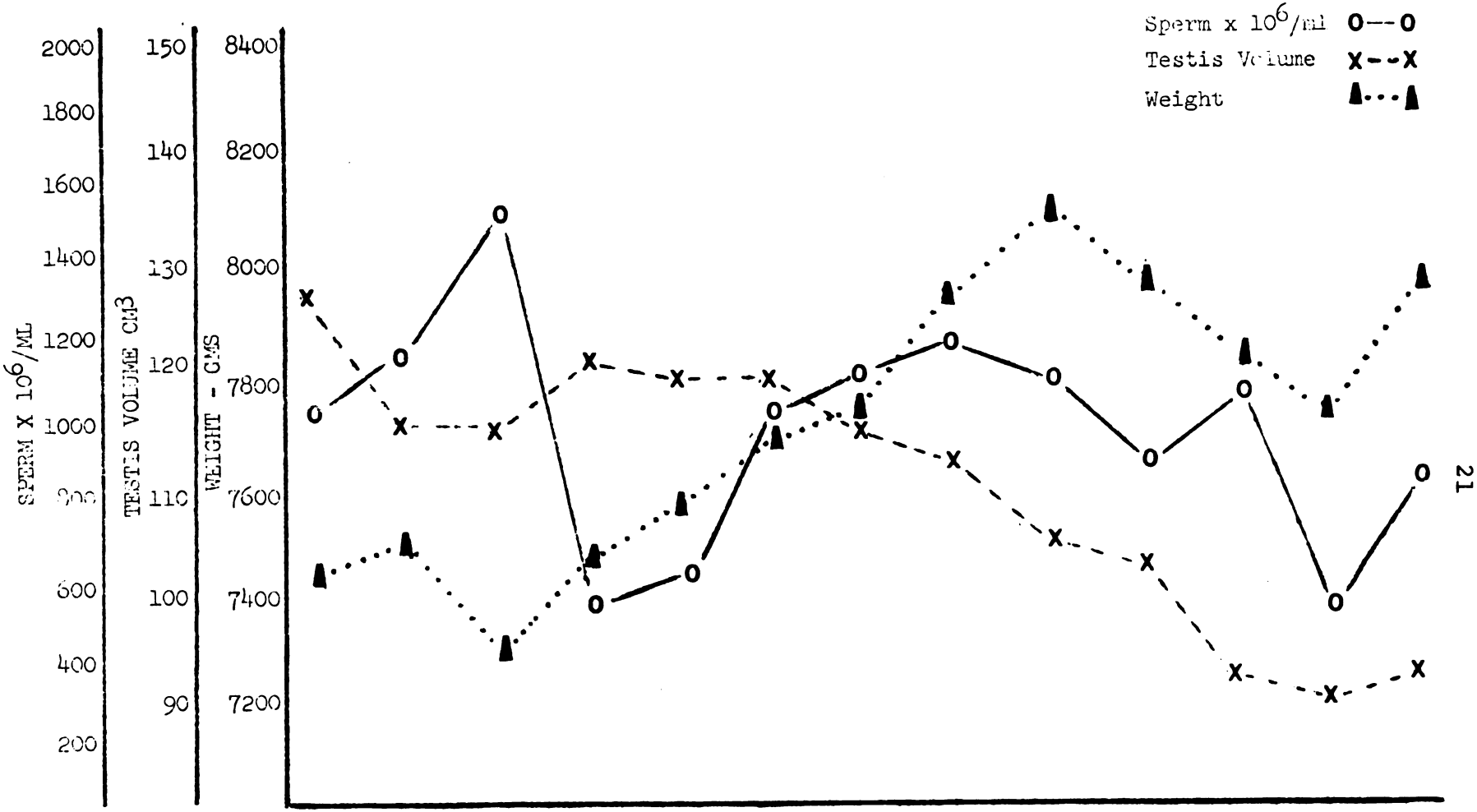
Neither comparison for the months of high versus low body weights or breeding versus nonbreeding season were statistically different ($p > 0.05$).

Average values for all three criteria examined over the thirteen 28 day intervals are shown in Figure 4. Since no statistically significant change in either sperm concentration or body weight occurred, no attempts at correlating either of these characteristics with each other or with testis volume were made.

Discussion

The existence of an annual rhythm in sexual performance and motivation, as distinguished from breeding, has been well-documented in the laboratory housed male rhesus (M. mulatta). This rhythmicity is extremely persistent in the absence of photoperiod and temperature fluctuation in the intact male as androgen rhythms continue under these conditions (Michael and Zumpe, 1976; Michael and Bonsall, 1977). Annual changes in behavioral performance in the castrate male can occur in the absence of any major changes in plasma androgen levels (Michael et al., 1975). Michael and Wilson (1975) demonstrated that castrated males receiving exogenous testosterone, and paired with ovarioectomized estrogen treated females continued the same annual rhythm in mating performance as did intact males, although the annual changes

Figure 4. Mean values for changes in sperm concentration, average testis volume and body weight over a 13 lunar month period in laboratory housed male, Macaca fascicularis.



LUNAR MONTHS

Figure 4

were less dramatic than for intact males.

The behavioral status of the rhesus is markedly different than that of the laboratory housed male M. fascicularis. Unpublished observations from our laboratory indicate that male cynomologus macaques are sexually active and the females receptive and capable of conception during every month of the year.

Valerio (1969) has reported a very rapid decrease in the annual reproductive cycle of M. fascicularis which reflects on both male reproductive efficiency as well as that of the female.

Analysis of data from the present study demonstrates that a definite seasonal effect on reproduction exists as far as changes in testicular size are concerned. The importance of this effect to the overall reproductive efficiency of the male is uncertain, particularly in light of the lack of corresponding significant change in sperm concentration or body weight for either high mean vs. low mean intervals or during the "standard" breeding season. It is interesting that the smallest testicular volume in M. fascicularis occurs during the fall-winter months. This is the period during which maximal volumes would normally be expected in the rhesus. The most important parameter, sperm concentration, shows no statistical variation. This, when considered with lack of rhythmic changes in body size, would indicate a

stability of annual male reproductive characteristics not exhibited by M. mulatta.

It is probable that any changes in reproductive patterns would be reflected in hormonal variation.

Increases in androgen levels associated with reproductive function could potentially be influenced by factors other than seasonal triggers. The effects of ejaculation on testosterone levels in M. arctoides (Goldfoot et al., 1975) and M. mulatta (Phoenix et al., 1977) have been examined. In neither species were testosterone levels elevated. Testosterone concentrations were monitored in these experiments for several hours after ejaculation. This indicates that stimuli other than mating would bring about increases in testosterone during the breeding season.

Position in the primate social order appears to have an extremely important influence on testosterone levels. Rose et al. (1975) observed a fall in testosterone levels in adult male rhesus following fighting defeat while the assumption of alpha (highest social) status led to an increase in testosterone levels. It was proposed that defest produced a fall in testosterone with the lower androgen concentrations decreasing the probability of aggressive action on the part of the subject. This would reduce the possibility for instigation of further combat and repeated defeats.

A complete evaluation of persistence of seasonal reproductive variation in M. fascicularis must include determination of total androgen or testosterone levels. Full clarification of the environmental cues responsible for triggering seasonal variation must include rigid control of humidity as well as photoperiod and temperature.

Based on the present study it appears that an attenuation of the variability of annual reproductive characteristics occurs in the laboratory housed male M. fascicularis. This species is, therefore, a much more suitable model for reproductive studies related to the human than the more commonly used M. mulatta.

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2) SEMEN COLLECTION AND PRESERVATION IN *MACACA FASCICULARIS*

SEMAN COLLECTION AND PRESERVATION IN MACACA FASCIAULARIS

Abstract

Semen was collected from adult male M. fascicularis using a rectal probe for electroejaculation. Power was supplied to the probe by a square wave generator. Pulse width ranged from 1.8 to 7.0 msec, pulse frequency from 35 to 75 Hz with a maximum voltage output of 40 volts. The effect of varying semen extender egg yolk concentration, pH, glycerol concentration and equilibration times of sperm with glycerol or sperm motility was examined. No significant difference was observed between motilities at extender egg yolk concentrations of from 10 to 40%. Progressive motility was significantly greater at pH's of 7.2 and 8.0 than at 5.8, 6.5 and 8.7 ($p < 0.05$). Glycerol concentrations of 7 and 10% yielded optimum progressive motility after freezing. A one minute equilibration of extender containing glycerol resulted in greater sperm motility after freezing than did equilibration with glycerol for 25 or 45 minutes.

Introduction

Nonhuman primates are widely used in biomedical research because of their physiological and anatomical similarity to humans. Animals used as research subjects often have no prior health records or information on their genetic composition.

Many benefits could be derived from a successful semen collection and preservation program to be utilized with artificial insemination for increasing the production of nonhuman primates in captivity. Semen from males selected for possession of superior characteristics of high heritability or for possession of traits valuable for the study of inherited defects could be disseminated throughout the research community. Seasonal declines in sperm production could be obviated through successful semen preservation and insemination techniques. Reduction in the total number of males required to be maintained for breeding purposes would be possible.

Semen Collection

Theoretically a wide variety of semen collection techniques could potentially be used with nonhuman primates. The artificial vagina is commonly used with domestic livestock. Attempts to use this technique with nonhuman primates have yielded little success (D. D. Hoskins, personal communication). Roussel and Austin (1968) reported little success

in collecting semen from nonhuman primates by masturbation or through stimulation with a massage vibrator. Semen has been collected from the vaginas of naturally mated female cynomologus macaques (Cho and Honjo, 1973). There are several disadvantages to this procedure. First some of the semen may be diluted and contaminated by vaginal secretions. Another factor to be considered is the adverse effect of the female tract environment on sperm. Finally, the use of this procedure necessitates the use of additional receptive females to be maintained for collection.

Electroejaculation is the technique most commonly used for semen collection in nonhuman primates. With smaller monkeys this technique can be successfully employed without anesthesia (Kuehl and Dukelow, 1974). Mastroianni and Manson (1963) have reported successful recovery of semen following electroejaculation of unanesthetized macaques with penile electrodes.

The more common method involves electroejaculation of anesthetized animals with rectal electrodes. Rectal probes have been effectively employed in a wide variety of nonhuman primates (Fussel et al., 1967; Roussel and Austin, 1968; Kraemer and Vera Cruz, 1969). Warner et al. (1974) investigated different current flow patterns and obtained optimum collection using a circular pattern provided by longitudinal electrodes.

The ejaculate of the nonhuman primate is generally characterized by formation of a coagulum or plug that may be accompanied by a liquid fraction. Different enzymatic agents have been used to reduce or prevent coagulum formation and to increase the number of spermatozoa in the liquid fraction (Hoskins and Patterson, 1967; Van Pelt and Keyser, 1970; Bush et al., 1975). The disadvantage of this approach lies in the unknown effects of the various enzymes on sperm fertility.

Semen Preservation

The discovery of Polge et al. (1949) that glycerol protects spermatozoa during the freezing process has led to the extensive use of frozen semen in domestic and exotic animal breeding as well as in humans. The success of freeze preservation is based on three factors: 1) the critical temperature ranges through which the spermatozoa pass before reaching storage temperature; 2) the rate at which the semen is cooled, and 3) the cryoprotective agent in which the semen is extended (Ackerman and Behrman, 1968).

As spermatozoa are cooled they are subjected to cold shock. This occurs as the semen is cooled from room temperature to about +5°C and is characterized by a rapid and permanent loss of motility, decreased rate of metabolism and decreased fertilizing capacity. Cold shock appears to increase the permeability of the sperm cell (Mann, 1949).

The latent heat of crystallization is reached at from +5°C to -5°C and is associated with initial ice crystal formation. The sperm are subjected to a sharp temperature increase at this point. Further crystallization occurs down to -15°C.

The zone of recrystallization falls between -15°C and -79°C. It is this range that the growth and reunion of ice crystals into larger complexes occurs. Under any storage system there is wide enough fluctuation in temperature to allow for recrystallization. Once semen has been preserved, an assessment of the efficiency of the technique used is essential.

The absolute criterion for evaluation of successful semen preservation is the attainment of pregnancy. This is difficult in nonhuman primate research colonies where relatively few animals may be available for insemination. Other biochemical and morphological measures have been used in situations where indirect evaluation of sperm activity is required.

Nigrosin-eosin (live-dead) staining, in which dead spermatozoa take up the stain, has been used to evaluate sperm viability. Mittal (1975) has recently reviewed this area. The efficiency of live-dead staining is usually compared with motility for assessing semen quality.

Assay techniques for sperm enzymes have been employed in a variety of different situations based on the premise that as the sperm cell is frozen its cellular membranes are damaged, resulting in the release of cellular material (DeReuck and Knight, 1964; Brown et al., 1971; Stambaugh and Buckley, 1968; Zaneveld et al., 1972; Foulkes and Watson, 1975; Schill, 1975). Morphological changes in the acrosome during freezing also show promise as a predictor of potential fertility (Saacke and White, 1972; Pursel et al., 1972).

The most rapid and reliable method of assessing the viability of spermatozoa is progressive or forward motility. The correlation with fertility is low.

Traditional attempts to successfully freeze nonhuman primate semen have relied on techniques developed for use in domestic animals. Equilibration for periods up to several hours with or without glycerol at 4-5°C, followed by controlled cooling to storage temperature or rapid pellet freezing on dry ice have all been successful.

Good motility has been obtained after freezing in extenders containing egg yolk, glycerol, citrate and phosphate buffers, following incubation at 37°C for 30 minutes in Macaca mulatta (Leverage et al., 1972). Roussel and Austin (1967) achieved good past thaw motility in an extender consisting of egg yolk, sodium glutamate buffer and 14% glycerol. Kraemer and Vera Cruz (1969) utilized this same extender,

plus penicillin and streptomyocin, to successfully freeze Papio sp semen.

An extender consisting of glucose, lactose, raffinose and 5% glycerol has been used to pellet freeze Macaca fascicularis semen. This extender was maintained at a pH of 7.2 with tris buffer. Dennis et al. (1976) have used a two step equilibration period with extender containing 4% glycerol, egg yolk and lactose to pellet freeze squirrel monkey semen.

The basic extender components usually include egg yolk and glycerol. Lactose has been demonstrated to be a non-penetrating cryoprotectant and is highly beneficial in any rapid freezing technique (Rowe, 1966). pH level is normally maintained in the physiological range. The discovery that egg yolk has a beneficial effect by diminishing cold shock (Phillips, 1939) has led to its widespread use in semen extenders. Pace and Graham (1974) have demonstrated that semen extenders containing 20% egg yolk, in the absence of glycerol, protected the motility of bull sperm during the freezing process. Berndston and Foote (1972) have demonstrated that exposure of bull semen to glycerol for as short as ten seconds is sufficient for successfully freezing, indicating that glycerol may produce its effect very rapidly.

The objectives of the present study were:

1) To evaluate the efficacy of electroejaculation for semen collection in M. fascicularis.

2) To examine the effect of egg yolk concentrate on the viability of sperm when frozen.

3) To determine the effect of varying pH levels on the viability of sperm subjected to freezing.

4) To determine if glycerol is required for the successful pellet freezing of semen and at what levels.

5) To determine if a short equilibration with glycerol prior to pellet freezing is as effective as longer equilibration periods.

Materials and Methods

Semen Collection

A rectal probe designed after Healy and Sadlier (1966) was used for electroejaculation. Power was supplied to the probe by a square wave generator. Pulse width ranged from 1.8 to 7.0 msec, pulse frequency 35 to 75 Hz and maximum voltage output was 40 volts.

A sling was used to support the animal during semen collection (Roussel and Austin, 1968).

Seven adult M. fascicularis males were used to develop the semen collection technique. The animals were individually housed and received a commercially prepared monkey diet (Wayne; Allied Mills Inc.) and water ad libitum. Following an intramuscular injection (10 mg/kg body weight) of

Ketamine-HCL (Vetalar, Parke-Davis) to induce anesthesia, the monkey was positioned in the support sling. Its feet were secured to the rear posts of the frame and the rectal probe inserted so that the last brass ring was just inside the anal sphincter.

Stimulation was accomplished by gradually increasing the voltage in an on-off pattern lasting from 2-5 seconds. The pattern was maintained until ejaculation occurred. Semen was collected in a 3 ml plastic vial and placed in an incubator at 37°C for 30 minutes. After incubation, the semen volume was measured and the sperm concentration determined with a hemocytometer. The percent progressive and total motility was determined after dilution (1:20) in physiological saline warmed to 37°C. At least 100 sperm were counted and evaluated for motility. Progressive motility was defined as rapid forward motion of individual sperm cells while total or raw motility was comprised of total motile sperm, regardless of direction or speed of movement.

Semen Preservation

A single extender was used for the semen freezing experiments. The basic extender formula consisted of 4% glycerol, 20% fresh egg yolk, and 11% lactose in distilled water with individual variations depending on the variable to be tested. The pH was adjusted, dependent on experiment, with 4% tris buffer.

Following collection and equilibration of the semen at 37°C, the whole ejaculate was maintained at room temperature for 10 minutes while sperm concentration, progressive and total motilities were determined. The ejaculate was split into 10 µl aliquots and at the end of equilibration at room temperature, 45 µl of semen extended was added at 4°C. The extended semen was then equilibrated at 4°C for 20 minutes. An additional 90 µl of extender was added and equilibrated with the semen for 25 minutes before pellet freezing on dry ice.

After freezing, the semen was incubated at 37°C for 15 minutes. The percent of original progressive and total motility was then determined.

Experiment 1--Egg Yolk Concentration

The extender used was composed of 4% glycerol, 11% lactose and was buffered to a pH of 7.2 with 4% tris buffer. Egg yolk concentration was 10, 20, 30 or 40%. Ejaculates were collected from four males. Following collection, the semen was equilibrated with extender and pellet frozen as previously described. The data were analyzed using Dunnet's t-test.

Experiment 2--pH Levels

The extender consisted of 4% glycerol, 11% lactose, 20% egg yolk. It was buffered to a pH of 6.5, 7.2, 8.0 or 8.7

with 4% tris buffer. The pH of unbuffered extender was left at 6.0. Four separate semen collections were made from each of four males. Semen was equilibrated with extender and pellet frozen on dry ice. Since a wide pH range has been used to freeze primate semen, Scheffe's interval was used to analyze the data collected.

Experiment 3--Glycerol Concentrations

An extender was formulated with 20% egg yolk, 11% lactose and adjusted to a pH of 7.2 following addition of glycerol. Glycerol levels used were 0, 4, 7, 10 and 20%. Semen from three males was obtained on each of four days. The semen was extended and frozen in the same manner as described for experiments 1 and 2. The data were analyzed using orthogonal f-tests for single degree of freedom contrasts.

Experiment 4--Glycerol Equilibration Time Before Freezing

An extender consisting of 20% egg yolk, 7% glycerol, 11% lactose and buffered to a pH of 7.2 with 4% tris buffer was formulated. Equilibration times of 1, 25 or 45 minutes with glycerol prior to freezing were examined for their effect on post thaw motility.

Four males were collected on each of four days.

The protocol used for equilibration with extender differed from that used in the first three experiments.

Semen equilibrated with glycerol for 1 or 25 minutes was first placed in extender containing no glycerol for 20 minutes. At the end of this period, extender containing 14% glycerol (to bring the final extender concentration to 7%) was added and equilibrated at 4°C for 1 or 25 minutes before pellet freezing on dry ice. The 45 minute treatment group was handled in the same way as in the first three experiments. Specifically, extender containing 7% glycerol was added during both equilibration periods of 20 and 25 minutes. In all 3 treatment groups, the final extender composition was 20% egg yolk, 11% lactose and 7% glycerol at a pH of 7.2. Orthogonal f-tests for single degree of freedom contrasts were used to analyze the data.

Results

Semen was collected from seven male cynomologus macaques using a rectal probe for electroejaculation. Of 410 trials, 407 resulted in successful collection of ejaculates. The three unsuccessful attempts were a result of stimulator malfunction.

Stimulation with the rectal probe for from 2 to 10 minutes resulted in good recovery of ejaculate volume. The volume of the liquid parts of the sample following incubation at 37°C for 30 minutes, ranged from 10 µl to 1.5 ml.

Sperm concentration varied from 0 to 5 X 10^9/ml with progressive motility values from 0 to 97%.

Four males that had consistently yielded high concentration (in excess of 20 X 10^6/ml) following electroejaculation were selected for use in subsequent semen freezing experiments.

Experiment 1

Mean values for collections from each male, reflecting the effect of egg yolk concentration on the sperm motility after freezing, are shown in Table 1. The effect of this treatment, expressed as a percent of original progressive and original total motility, is depicted in Table 2. There was no statistical difference ($p > 0.05$) between the percent of original progressive or total motility for any of the egg yolk concentrations examined.

Experiment 2

The effect of extenders buffered to varying pH's is shown in Table 3. Motility is expressed as percent original progressive and total motility in Table 4. Progressive motility was significantly greater at pHs of 7.2 and 8.0 than at 5.8, 6.5, or 8.7. Total motility was significantly greater ($P < 0.05$) at pH 8.0 when contrasted with that at pH 8.7 but not between 8.0 and any of the other treatment groups examined ($p > 0.05$).

Table 1. Effect of Egg Yolk Concentration on the Motility of Macaque Sperm After Freezing

Male Number	Original Motility Before Extending	Egg Yolk Concentration 10%	20%	30%	40%
		Percent Progressive Motility			
1	30	20	21	17	29
2	75	0	11	10	23
3	75	20	38	43	50
4	18	4	5	24	8
$\bar{X}\pm$SE (n=4)	49.5±14.9	11.0±5.3	18.8±7.2	23.5±7.1	27.5±8.7
		Percent Total Motility			
1	35	35	36	25	36
2	81	25	22	20	54
3	81	30	48	60	69
4	31	9	23	41	21
$\bar{X}\pm$SE (n=4)	57±13.9	24.8±5.6	32.3±6.1	36.5±9.0	45.0±10.5

Table 2. Effect of Egg Yolk Concentration as a Percent of Original Motility of Macaque Sperm After Freezing

Male Number	Egg Yolk Concentration 10%	20%	30%	40%
		Progressive Motility		
1	67	70	57	97
2	0	15	13	31
3	27	51	57	67
4	22	28	100	44
$\bar{X} \pm$ SE	29±18.9	41±23.1	57±32.3	60±32.4

Statistical Analysis for Progressive Motility

10% vs. 20% ($p > 0.05$)
10% vs. 30% ($p > 0.05$)
10% vs. 40% ($p > 0.05$)

		Total Motility		
1	100	100	71	97
2	31	27	25	67
3	37	59	74	85
4	29	74	100	68
$\bar{X} \pm$ SE	49±28.7	65±35.1	68±36.4	79±40.1

Statistical Analysis for Total Motility

10% vs. 20% ($p > 0.05$)
10% vs. 30% ($p > 0.05$)
10% vs. 40% ($p > 0.05$)

Table 3. Effect of pH Level on the Motility of Macaque Sperm After Freezing

Male Number	n	Original Motility Before Extending	pH 6.0	6.5	7.2	8.0	8.7
			Percent Progressive Motility				
1	4	7.6±1.4	0.1±0.1	0.1±0.1	0.2±0.2	2.0±0.1	2.0±2.0
2	4	39.2±5.3	3.0±2.1	4.9±1.4	7.5±1.6	6.3±1.7	1.4±1.0
3	4	40.1±7.2	0.0±0.0	0.7±0.7	5.4±1.9	15.7±3.0	2.8±0.5
4	4	64.9±9.4	5.4±3.2	6.5±4.0	14.2±7.6	15.7±5.7	2.2±1.4
$\bar{X}$ ± SE	16	(38.1±6.0)	(2.1±1.0)	(3.0±1.2)	(6.8±2.2)	(9.8±2.2)	(2.1±0.6)
			Percent Total Motility				
1	4	19.7±5.0	4.8±2.5	4.3±1.8	2.3±0.9	28.0±24.0	1.6±1.0
2	4	64.0±8.3	15.7±5.1	17.8±2.0	19.7±3.1	18.8±4.1	19.2±4.0
3	4	58.0±7.2	7.8±4.5	20.4±7.9	15.2±0.5	33.6±4.9	15.2±3.0
4	4	82.2±7.5	32.1±13.1	38.1±6.0	41.5±6.5	34.9±7.2	19.1±7.7
$\bar{X}$ ± SE	16	56.0±6.7	15.1±4.3	20.2±3.9	19.7±4.0	28.8±6.0	13.8±2.8

Table 4. Effect of pH Level as a Percent of Original Motility of Macaque Sperm After Freezing

Male Number	(n)	pH Level 6.0	6.5	7.2	8.0	8.7
			Progressive Motility			
1	4	3.6±1.9	4.2±2.4	5.5±3.4	31.5±18.8	0.0±0.0
2	4	9.6±6.3	14.2±4.5	22.0±6.1	18.1± 6.4	4.7±3.3
3	4	0.0±0.0	1.8±1.3	14.9±2.9	43.5±12.3	8.9±2.8
4	4	8.1±4.7	9.8±5.0	20.6±10.8	29.6±13.5	3.5±1.7
$\bar{X}$ ± SE	16	5.3±2.3	7.3±2.5	15.7±4.8	30.7±9.4	4.3±1.6
			Statistical Analysis			
			8.0 v. 6.0 ($p < 0.05$)			
			8.0 v. 6.5 ($p < 0.05$)			
			8.0 v. 7.2 ($p > 0.05$)			
			8.0 v. 8.7 ($p < 0.05$)			
			Total Motility			
1	4	22.2±7.8	25.1±13.0	16.8±6.5	42.0±20.3	1.4±6.8
2	4	23.4±5.3	28.9±2.5	31.2±2.9	32.4±10.1	30.0±3.5
3	4	12.6±7.1	33.8±12.6	28.3±4.8	62.9±13.9	28.5±6.3
4	4	36.8±15.3	47.3±6.6	51.6±6.9	42.6±6.7	22.1±9.0
$\bar{X}$ ± SE	16	23.7±7.1	33.8±8.9	32.0±8.1	45.0±12.0	23.0±6.2
			Statistical Analysis			
			8.0 v. 6.0 ($p > 0.05$)			
			8.0 v. 6.5 ($p > 0.05$)			
			8.0 v. 7.2 ($p > 0.05$)			
			8.0 v. 8.7 ($p < 0.05$)			

Experiment 3

Sperm motilities after equilibration in extenders containing 0, 4, 7, 10 and 20% glycerol are given in Table 5. The percent of original motility after freezing is depicted in Table 6. Progressive motilities in extenders containing glycerol at 7 and 10% concentrations were significantly greater (p 0.05) than at 4%. There was no significant difference (p 0.05) between progressive motilities for extenders containing 7 and 10% glycerol concentrations nor between total motilities at 7 and 10% glycerol concentrations. Total motility was greater at 7 and 10% glycerol than at 4 and 20% (p 0.05). Extender containing no glycerol resulted in no progressive or total motility and was excluded from the statistical analysis.

Experiment 4

Table 7 shows the effect on motility of time of glycerol equilibration prior to pellet freezing. The effect of equilibration with glycerol for 1, 25 or 45 minutes prior to pellet freezing on the percent of original progressive and total motility is demonstrated in Table 8. Progressive motility was significantly greater (p 0.05) after one minute equilibration than following 25 or 45 minutes of equilibration with glycerol. No significant differences (p 0.05) was observed between progressive motilities at 25 or 45% nor between total motilities for any of the equilibration times examined.

Table 5. Effect of Glycerol Concentration on the Motility of Macaque Sperm After Freezing

Male Number	n	Original Motility Before Extending	Glycerol Concentration $\bar{X}$ + SE 0%	4%	7%	10%	20%
			Percent Progressive Motility				
1	4	23.9±3.5	0.0±0.0	1.1±0.6	3.4±1.5	4.1±1.9	0.0±0.0
2	4	66.7±6.2	0.0±0.0	6.1±3.0	6.0±3.2	11.0±3.7	0.6±0.6
4	4	54.2±9.0	0.0±0.0	1.3±1.3	10.8±7.9	11.5±6.7	0.0±0.0
$\bar{X}$ ± SE	12	48.3±6.4	0.0±0.0	2.8±1.3	9.1±2.8	8.9±2.6	0.2±0.2
			Percent Total Motility				
1	4	55.7±6.7	0.0±0.0	7.4±1.8	16.2±4.3	22.5±4.3	1.0±1.0
2	4	77.3±3.4	0.0±0.0	30.0±6.8	33.9±1.2	23.6±2.3	4.6±1.6
4	4	63.6±7.4	0.0±0.0	32.0±9.0	40.1±11.4	46.6±13.6	9.8±5.4
$\bar{X}$ ±SE	12	65.5±4.1	0.0±0.0	23.3±4.8	30.1±4.8	30.9±5.7	5.1±2.0

Table 6. Effect of Glycerol Concentration as a Percent of Original Motility of Macaque Sperm After Freezing

Male Number	n	Glycerol Concentration 0%	4%	7%	10%	20%
			Progressive Motility			
1	4	0.0±0.0	7.5±4.1	16.1±6.7	17.6±6.3	0.0±0.0
2	4	0.0±0.0	9.6±5.1	20.2±3.8	17.3±6.3	0.0±0.0
4	4	0.0±0.0	5.1±4.6	17.5±11.7	19.5±11.0	0.0±0.0
$\bar{X}$ ± SE	12	0.0±0.0	7.4±3.0	18.0±6.1	18.1±6.2	0.0±0.0
			Statistical Analysis			
			7% v. 10% ($p > 0.05$)			
			4% v. 7%, 10% ($p < 0.05$)			
			Total Motility			
1	4	0.0±0.0	22.1±3.7	28.6±4.9	38.4±7.1	2.0±1.5
2	4	0.0±0.0	40.2±9.1	44.5±1.6	31.3±3.5	6.4±2.0
4	4	0.0±0.0	52.0±14.2	63.9±19.0	70.1±17.2	15.0±8.0
$\bar{X}$ ± SE	12	0.0±0.0	38.1±11.3	45.7±13.2	46.6±13.5	7.8±3.4
			Statistical Analysis			
			7% v. 10% ($p > 0.05$)			
			7%, 10% v. 4%, 20% ($p < 0.05$)			

Table 7. Effect of Glycerol Equilibration Time on the Motility of Macaque Sperm After Freezing

Male Number	n	Original Motility Before Extending	Glycerol Equilibration Time 1 minute	25 minutes	45 minutes
			Percent Progressive Motility		
1	4	30.5±6.7	10.1±2.9	8.8±4.2	7.9±3.0
2	4	51.1±6.0	19.9±7.6	4.6±4.5	12.3±5.1
3	4	47.6±4.9	23.5±4.0	13.6±4.0	14.6±2.3
4	4	38.2±13.3	12.1±4.5	7.9±2.7	1.5±1.5
$\bar{X}$ ± SE	16	41.8±4.3	16.4±2.7	8.7±1.9	9.1±1.9
			Percent Total Motility		
1	4	60.6±4.5	24.8±8.5	22.8±5.4	23.3±3.5
2	4	84.4±1.0	52.5±4.6	41.8±5.4	37.9±7.6
3	4	74.6±5.2	47.5±6.5	39.7±5.4	27.9±5.4
4	4	49.7±14.4	29.5±4.4	30.1±5.7	22.7±8.1
$\bar{X}$ ± SE	16	67.3±4.9	38.6±4.1	33.6±3.1	28.0±3.3

Table 8. Effect of Glycerol Equilibration Time as a Percent of Original Motility of Macaque Sperm After Freezing

Male Number	n	Glycerol Equilibration Time 1 Minute	25 Minutes	45 Minutes
		Percent Progressive Motility		
1	4	36.5±10.7	29.2±16.4	28.7±11.6
2	4	35.8±12.4	9.6±9.1	24.0±10.4
3	4	49.2±4.9	27.5±5.1	30.7±2.4
4	4	46.7±22.2	33.7±18.0	5.8±5.3
$\bar{X}$ ± SE	16	42.0±11.4	25.0±8.4	22.3±6.6
		Statistical Analysis		
		1 min > 25 min, 45 min ($p < 0.05$)		
		25 min v. 45 min ($p > 0.05$)		
		Percent Total Motility		
1	4	39.8±11.2	38.2±15.5	38.7±3.6
2	4	62.8±5.7	49.2±5.8	45.7±9.4
3	4	64.8±8.2	55.4±9.7	37.9±6.9
4	4	70.1±13.6	72.1±16.4	60.3±24.3
$\bar{X}$ ± SE	16	59.4±14.3	53.7±13.3	45.6±12.1
		Statistical Analysis		
		1 min v. 25 min, 45 min ($p > 0.05$)		
		25 min v. 45 min ($p > 0.05$)		

Discussion

The semen collection trials demonstrated that electroejaculation is a rapid and reliable method for obtaining spermatozoa from M. fascicularis. The stress associated with extensive anesthesia and electroejaculation is minimal as is evidenced by continued good health and weight maintenance during these experiments. In view of these results, rectal electroejaculation is the method of choice for semen collection in this nonhuman primate.

The results of Experiment 1 indicate that a wide range of egg yolk concentrations are effective as cryoprotectants in the semen preservation process. Virtually all semen extenders contain egg yolk as a constituent, but no additional benefit appears to be derived from increasing the concentration above 20% in M. fascicularis.

The results of the second experiment demonstrate that extenders in the pH range of 7.2 to 8.0 provide the better post thaw motility. It is surprising that pH 8.0 would be so effective, since extenders used previously for nonhuman primate semen preservation have normally been adjusted to pH 7.2 (Cho and Honjo, 1973) or left at pH 6.0 (Denis et al., 1974). Meizel and Mukerji (1975) have reported the greatest activation of proacrosin to its active form acrosin is at pH 8.0. Acrosin is involved in the penetration of the zona pellucida by the sperm. The greater motility obtained in

the pH range 7.2-8.0 may be related to this phenomenon in that the higher pH results in increased metabolic activity associated with motility.

The effects of glycerol levels as a cryoprotectant for macaque sperm during the freezing process are very straight forward. The data show a definite requirement for glycerol as no motility was obtained after freezing when glycerol was omitted from the extender. Glycerol levels similar to those used with human semen (7-10%) were more effective than those used for the squirrel monkey (Denis et al., 1974). The high glycerol level (20%) was detrimental to spermatozoa probably due to osmotic effects of the high concentration of the solution.

The final investigation (Experiment 4) demonstrated that, while glycerol is a required extender constituent for freeze preservation, a very short equilibration time with glycerol prior to freezing results in maximum motility after freezing. Berndston and Foote (1972) working with bull semen, have suggested that glycerol has an immediate dehydration effect on sperm cells and that rapid freezing decreases the effect of intracellular ice crystal formation and avoids the effect of high osmotic pressure. An alternate hypothesis is that glycerol may act as a sperm membrane stabilizer to protect the cell during freezing but that longer incubation may exert an osmotic influence resulting in cell damage.

Both the dehydration and membrane stabilization effect may act simultaneously to protect the individual sperm.

While insemination with fresh or extended seman has been successful, no nonhuman primate inseminated with frozen semen has given birth to live young. Leverage et al. (1972) artificially inseminated M. mulatta with frozen semen. Of 48 inseminations, only one resulted in pregnancy. This conceptus was aborted at 40 days of gestation. Cho et al. (1975) have obtained 8 successful pregnancies following 13 artificial inseminations in 8 animals with frozen semen. These pregnancies resulted in abortion 6-8 weeks after conception.

The result of the present study indicates the value of examining different aspects of extender components and freezing techniques. Future studies should examine all variables necessary to promote optimum motility in lieu of making direct application of successful methods in one species to nonhuman primates. These studies must eventually include insemination trials to assess fertility of the frozen semen.

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3) REPRODUCTIVE PERFORMANCE IN *MACACA FASCICULARIS* FOLLOWING EXTENSIVE LAPAROSCOPY

REPRODUCTIVE PERFORMANCE IN *MACACA FASCICULARIS* FOLLOWING EXTENSIVE LAPAROSCOPY

Abstract

Thirty-six mating trials were conducted over an eleven month period with sixteen adult female *M. fascicularis* that had been extensively laparoscoped for characterization of follicular morphology and determination of ovulation. No difference was observed in menstrual cycle length when laparoscopy was performed versus cycles during which no laparoscopic examinations were conducted. Five (31.3%) of the females in this study conceived. Three gave birth to healthy live infants. Two abortions from unknown causes occurred at 112 and 123 days gestation to animals which had been laparoscoped 43 and 54 times previously. Three females delivering live infants had been laparoscoped 38, 47 and 49 times prior to breeding.

Introduction

The present cost of captured primates precludes the practical use of captive bred animals for research. The continued depletion of feral populations coupled with stringent export restrictions by exporting countries increases the necessity for captive breeding programs. Numerous factors influence the reproductive performance of captive subhuman primates. Included among these are adaptation to the captive environment, seasonal effects, and the type of mating strategy.

Hendrickx and Kriewaldt (1969) reported that an eight month adaptation time was required before menses reappeared following introduction of baboons (Papio spp) into the laboratory. Harrison and Dukelow (1973) observed a nine month adaptation period after introduction to the colony before squirrel monkeys (Saimiri sciureus) would respond to an ovulatory regime. An increase in conception rate following increasing length of residence in captivity has been reported for three species of macaques (Valerio et al., 1975).

Seasonal effects on reproduction are extremely pronounced in feral primate groups. Carryover of this effect to captivity has been observed for several years and can significantly contribute to decreased birth rate in captivity (Niemann, 1970).

The length of time that the female is housed with the male influences the conception rate. Grauwiler and Bruggemann (1972) reported a pregnancy rate of 5.1 exposures per conception using stumptailed macaques (Macaca arctoides) in timed mating trials with male to female exposure up to one hour. Macdonald (1971) obtained M. arctoides pregnancies after a mean of 1.8 ± 0.3 exposures when a five day exposure time was allowed.

Many nonhuman primate cycles are characterized by the lack of any outward sign of estrus. Of extreme importance in any timed-mating research breeding program is the ability to accurately predict ovulation. Follicular morphological development and ovulation has been characterized through laparoscopic observation in the cynomologus macaque (M. fascicularis) (Jewett and Dukelow, 1972; Rawson and Dukelow, 1973). Data available on the effect of extensive laparoscopy on the ability of nonhuman primates to conceive and deliver healthy infants is limited. Rawson and Dukelow (1973b), reported the case of a single M. fascicularis subjected to extensive anesthesia, bleeding and laparoscopy over a 33 day period. Mating and conception occurred on the 17th day and laparoscopy was again performed on days 4 and 7 of pregnancy. A normal infant was delivered at term.

The objective of this study was to evaluate the effect of extensive laparoscopic examinations on subsequent reproductive performance in M. fascicularis.

Materials and Methods

Sixteen female M. fascicularis maintained at the Endocrine Research Unit (Michigan State University) were used in this study. They were maintained on a 12 hour light:dark cycle. Temperature was regulated between 21-26°C with relative humidity fluctuating between 40 and 60% depending on the season. All animals received a commercially prepared monkey diet (Wayne, Allied Mills Inc.) and had unlimited access to water.

The macaques were individually housed in 122 cm stainless steel, flush-type cages equipped with slotted floors, perches and squeeze back devices to facilitate handling. Menstruation had been recorded daily over the last 8 years.

Seven adult male macaques were screened for motile sperm concentrations obtained both through electroejaculation or through vaginal aspiration after mating. Based on this screening, five males were selected for use in this study.

For laparoscopic examination an intramuscular injection (10 mg/kg body weight) of Ketamine-HCl (Vetalar, Parke-Davis) was used to induce anesthesia. The monkeys were placed on an inclined table with their feet elevated and secured with adjustable straps (Jewett and Dukelow, 1973). The abdomen was shaved and prepared with benzalkonium chloride (Zepherian, Winthrop, New York). A trocar-cannula was inserted

through a 8-10 mm incision in the abdomen. Following insertion the trocar was withdrawn and replaced with the laparoscopic telescope (135°, 5 mm. pediatric model, Richard Wolf Co., Knittlingen, W. Germany). A flexible fiber optic cable connected to a Wolf model 4000 light source was used to illuminate the abdomen through the laparoscope. A Verres cannula was inserted 5-6 cm. lateral to the laparoscopic insertion point as a probe for manipulation of the ovaries. The abdomen was insufflated with 5% CO_2/air.

After completion of the examination, the Verres cannula and laparoscope were withdrawn leaving the cannula in place to allow escape of the insufflation gas. The cannula was then removed. Furacin powder was applied and the skin incision closed with a single suture (0000 chromic gut, Ethicon). Penicillin -G(150,000 units) was then administered to combat infection.

Laparoscopic observation of follicular development and the stage of the menstrual cycle dictated the time of introduction of each female into a cage with a male. Mating trials were conducted from as early as day 2 of the cycle until just before ovulation as determined by laparoscopic observation. Mating periods lasted from several hours to several days.

Results

Breeding trials were conducted over an eleven month period (Table 9). Five (31.3%) of the females conceived in 36 trials. Three females gave birth to healthy live infants. Two abortions occurred at 112 and 123 days of gestation to animals which had been laparoscoped 43 and 54 times previously. Gross anatomical observation of the fetuses did not reveal the cause of the abortion of these pregnancies. Three females delivering live infants had been Laparoscoped 38, 47 and 49 times prior to mating.

Discussion and Conclusion

Extensive use of laparoscopic examination of follicular development maximizes the utility of nonhuman primates for reproduction research. The technique of laparoscopy offers a feasible alternative to repeated laparotomy which results in abdominal adhesions after only 3 or 4 procedures. While adhesions can occur after extensive laparoscopy (generally in excess of 30) they are largely avoided by careful technique. Laparoscopic electrocoagulation and cautery can correct adhesions that do occur.

Laparoscopy is a reliable means of providing access to the internal organs of expensive primates such as the chimpanzee, enhancing the value of this experimental animal

Table 9. Summary of Results of Mating Trials Conducted Between May 1, 1975 and March 18, 1976

Animals	Number of Laparoscopic Observations	Mating Trials	Days of Exposure to Male Resulting in Pregnancy	Results
7	43	3	5	Abortion-(112 days
9	44	3		-
11	31	1		-
12	54	3	6	Abortion-(123 days)
17	45	2		-
31	32	1		-
39	47	1	6	Livebirth
40	67	1		-
41	38	2	6	Livebirth
42	44	2		-
43	47	3		-
44	64	6		-
51	82	2		-
53	49	2	4	Livebirth
54	43	3		-
55	32	1		-

model (Graham, 1976). Bosu (1973) observed no complications following over 100 laparoscopic examinations during the normal menstrual cycle of the rhesus monkey. Laparoscopic observation was used in 16 rhesus monkeys during 27 menstrual cycles (Dierschke and Clark, 1976). Cycle length was 27.5 ± 0.6 days in cycles during which laparoscopy was performed compared to 28.9 ± 0.6 days and 28.9 ± 0.8 days in cycles before and cycles after laparoscopy was performed (a non-significant difference). No difference was observed in the cycle length of control animals in which no laparoscopic examinations were conducted (27.8 ± 0.2 days). Laparoscopy also produced no change in the length of the follicular or luteal phase in animals undergoing laparoscopy when compared to control animals. This is in agreement with results reported by Dukelow (1977) in which the laparoscopic examination of M. fascicularis during 252 cyles resulted in a cycle length of 30.6 ± 5.0 (Mean ± S.D.) days was observed as compared to menstrual cycle length of 29.4 ± 11.1 days in 334 cycles in which no laparoscopies were performed.

The results of the present study demonstrate that non-human primates retain the capacity to conceive and deliver normal live young after extensive laparoscopic examinations. It is concluded that extensive laparoscopy does not interfere with the mechanism of ovulation, menstrual cycle length or normal pregnancy in M. fascicularis.

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SUMMARY AND CONCLUSIONS

Macaca fascicularis was used as a model nonhuman primate for investigation of seasonal effects on reproductive variables in the male. A technique for semen collection was developed and semen extender components and preservation techniques were altered to determine their effect on the motility of cynomologus macaque sperm after pellet freezing. Breeding trials were conducted to determine the effects of extensive laparoscopic examination on subsequent reproductive performance in the female M. fascicularis. The following conclusions are drawn from the experimental data obtained in these studies:

1. The effect of season on the annual variation of reproductive characteristics is markedly reduced in the laboratory housed male M. fascicularis.
2. Semen collection using a rectal probe for electroejaculation is a rapid, reliable method of obtaining motile spermatozoa for reproductive studies in the male cynomologus macaque.
3. A wide range of egg yolk concentration are efficient as cryoprotectants in semen preservation.

4. Semen extender pH in the range of 7.2 to 8.0 provide the best post thaw motility.

5. Cynomologus macaque sperm have a definite requirement for glycerol and that levels in the neighborhood of 7-10% are the most effective in maintaining motility after freezing.

6. Extremely short equilibration times of sperm with glycerol prior to freezing result in maximum sperm motility after freezing.

7. Extensive laparoscopy does not interfere with the mechanism of ovulation, menstrual cycle length or normal pregnancy in M. fascicularis.

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