OVARIAN AND UTERINE CHANGES OF GRAY SQUIRRELS

AS AFFECTED BY

SEASON, AGE, REPRODUCTIVE STATE AND EXOGENOUS HORMONES

by

Cleveland J. Cowles

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APPROVED:

Roy L. Kirkpatrick
R. L. Kirkpatrick, Chairman

Patrick Scanlon
P. F. Scanlon

H. S. Mosby

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INTRODUCTION

Sixty-two species of mammals, birds and fishes have become extinct in the United States since the seventeenth century. At least 109 vertebrate species are classified as endangered on the U.S. Department of the Interior's official list published in the Federal Register. Nineteen mammals including the Everglades fox squirrel (Sciurus niger avicennia) and the Kaibah squirrel (Sciurus kaibabensis) are considered threatened (Goodwin 1974). The Delmarva fox squirrel (Sciurus niger bryanti) is endangered as well (Taylor 1974). For a few endangered species, such as the Texas red wolf (Canis rufus) the protection afforded by wildlife refuges will probably be inadequate in preventing their disappearance. Gwynne (1974), quoting Seager, states that although there is no doubt that natural breeding is by far the best method of replenishment of endangered species, an unavoidable fact must be faced: in many cases it simply is no longer possible.

This suggests artificial methods of breeding as a possible alternative to extinction for certain wild species. However, it is well documented that the breeding of wild mammals in captivity for preservation, display or research is a major problem. The tree squirrels in particular have been shown to seldom breed in captivity (Crandall 1964) unless provided with large outdoor enclosures (Sanderson and Berry 1973). Studies of the induction of estrus and ovulation in the gray squirrel (Sciurus carolinensis) through application of exogenous hormones have been done by Mellace (1973) and Williams et al.

(unpublished). An objective of this project was to refine and further evaluate the techniques of induction of ovulation in the gray squirrel as described in those studies.

Due to its abundance, suppressed breeding in captivity, and taxonomic similarity to certain threatened species, the gray squirrel is an appropriate research model. The gray squirrel's susceptibility to stress (Guthrie et al. 1967) is of particular interest in such studies. The results of the research reported here will contribute to the knowledge needed for preservation of endangered species and the propagation of wild mammals used for display or research.

Information on the ovarian morphology of the sciurid and muroid rodents is available for less than 10 percent of the 230 recognized genera (Mossman 1966). To date, there has been only one descriptive study of the ovarian cycle of the gray squirrel. This was conducted in Great Britain by Deanesly and Parkes (1933). Another objective of the present research was to extend the findings of Deanesly and Parkes (1933) particularly in regard to quantitative aspects of follicular changes in the gray squirrel ovary. Emphasis was placed on the follicular changes as affected by season, age, and reproductive state using squirrels obtained in their native habitat at latitudes considerably different from those in the British study. Also, the information obtained was used as a standard of comparison for the ovarian activity of gray squirrels treated with exogenous hormones.

LITERATURE REVIEW

Use of Ovarian Analyses in Wildlife Management

The delineation of the breeding season of the gray squirrel is of major importance and is facilitated by study of the occurrence of estrus, parturition, lactation and other reproductive changes. The establishment of hunting seasons for the gray squirrel has long been a problem since its lactation period extends into the fall. The most prudent date to open a squirrel season would be when there are few lactating females in the population. In Virginia populations, this would occur in November since lactation peaks in October (Newell and Kirkpatrick 1968). However, because this would yield a lower harvest an earlier date is preferable. Although females still nursing second litters would be taken, other advantages of an early season overshadow these losses (Allen 1952). Uhlig (1955) and Redmond (1953) also favor an early season for similar reasons. Advantages of an earlier season also would be that it would allow harvesting of squirrels that may otherwise succumb to warbles and there is less opportunity for hunting to conflict with the onset of winter breeding (Redmond 1953, Uhlig 1955).

Complete understanding of the population dynamics of a species depends on productivity estimates. In some species, such as the beaver (Castor canadensis) or gray squirrel, the obtaining of accurate litter counts is difficult. Also the use of placental scar counts as an index of productivity has limitations since they do not reveal the extent of intrauterine mortality. Provost (1962) suggested the

use of a numerical relationship that would take into account these losses between ovulation and parturition. This relationship would be the ratio of luteal glands to number of fetuses (or young born). Once determined for a population, the number of young per 100 luteal glands could be used to estimate productivity of future samplings lacking fetal (or litter) counts. However, environmental conditions would have to be relatively similar between sampling periods.

The measurement of follicular development during comparable seasons of different years may indicate years of adverse environmental conditions affecting fecundity and explain lower incidence of luteal glands (Morrison 1960). In sheep (Ovis aries, Howland et al. 1966) and the cottontail rabbit (Sylvilagus floridanus, Kirkpatrick and Kibbe 1971) high nutritional plane is known to cause increased follicular development. This suggests that the ovary might be used as an index of nutritional condition under certain conditions.

Therefore the measurement of the development and occurrence of tertiary follicles and luteal glands can be considered a method of expanding the total knowledge of reproduction from ovarian analysis (Morrison 1960). A value of the use of ovarian structures such as luteal glands or corpora atretica as indices of productivity is that they are available before embryos can be readily detected in the one case and usually can be identified long after placental scars are gone in the other case (Provost 1962).

Environmental Factors Associated With Seasonal Reproductive Change

Early studies of the effects of hypophysectomy indicated the importance of the pituitary as a source of gonadotropin (Marshall and Amoroso 1960). There is good evidence that the secretion of these gonadotropins is mediated by the nervous system. Factors such as light, courtship behavior, confinement and nutrition influence the hypothalmic-pituitary axis via the nervous system and, in turn, reproduction (Marshall and Amoroso 1960). Sadlier (1969:104) in a thorough review of work on domestic animals and the ferret concluded that "all the evidence presented can be more validly interpreted as demonstrating that the seasonal nature of breeding in mammals is entirely dependent on responses to external environmental stimuli and there is therefore no need to postulate any inherent annual rhythm in the group." The concept of rhythmic variation in the intensity of continuous reproductive activity in preference to an all or none pattern is more widely accepted (Perry 1971).

It is difficult to substantiate theories on the control of seasonal breeding in wild animals simply because most studies of wild seasonal breeders are on field collected animals without corroboration by experimental laboratory analysis (Foreman 1962). Another problem of significance is that many studies so often are designed to isolate the role of a single factor that little is learned about the interaction of the many factors actually controlling the breeding season (Perry 1971).

Bissonette (1933), Woitkewitsch (1945), Hart (1951), Hammond

(1953), Thibault et al. (1966), and Davis and Meyer (1972) have demonstrated the importance of the effect of photoperiod on reproductive mechanisms. For example, at least 18 hours of continuous light are needed to induce breeding in the ferret (Putorius vulgaris) but breeding can also be initiated by exposing the animal to 12 hours of light per day plus one hour from midnight to 1:00 a.m. (Hart 1951). Also breeding can be stimulated by using a cycle of 4 hours of light and 20 hours of darkness interrupted with light between the 17th and 19th hour (Hammond 1953). Thus it appears that light sets a biological clock which in turn determines the period of sensitivity in which additional stimulation will result in a physiological response (Smith 1966).

There is a limited amount of evidence that the timing of the breeding season in sciurids is controlled partially by photoperiod. It is well known that two distinct periods of breeding activity occur annually in the gray squirrel. These consist of winter and summer breeding seasons, with slight variation in actual timing among areas as summarized by Mellace (1973). Like the gray squirrel, the flying squirrels (Glaucomys spp.) also have winter and summer breeding peaks (Hibbard 1935, Sollenberger 1943, Muul 1969a). Experiments have shown that many of the activity patterns of this species are controlled by light. Muul (1968) demonstrated that photoperiod (independent of temperature) triggers food storing in flying squirrels. The intensity of the activity was correlated with temperature. Daily activity patterns have also been shown to be regulated by photoperiod (Decoursey

1960). Muul (1969b) found that long photoperiod maintained during the normal winter breeding period inhibited testicular descent. Testicular descent was hastened by an accelerated decrease in photoperiod and reproduction was induced 6 months out of phase when animals were exposed to photoperiod 6 months out of phase. Also the normal annual cycle was maintained in absence of temperature cues.

Light has been shown to be of importance in at least one other species of tree squirrel. Woitkewitsch (1945) induced fertile matings in the European red squirrel (Sciurus vulgaris) after several years of breeding failure in captivity by gradually increasing the amount of light per 24 hours. Temperature, as implied above, probably plays a secondary role to light by controlling the intensity of breeding activity rather than the onset and duration of breeding in mammals (Sadlier 1969, Perry 1971). Sexual stimulation of young and adult male gray squirrels has been observed under increasing light and temperature and under decreasing light and temperature (Hoffman and Kirkpatrick 1959). Thus, they conclude that light or temperature is not solely responsible for sexual stimulation in the male.

The part played by nutrition in induction of seasonal breeding is difficult to evaluate in wild mammals since problems exist in assessing quantity and quality of available food (Sadlier 1969). Dietary factors so interact with each other and other factors such as climate that it is difficult to isolate causes and effects related to them (Perry 1971). Lampio (1967) suggested that the supply of spruce seed may have some influence on the number of litters produced by Sciurus vulgaris. Millar (1970) considered heavy snowfall as a

possible factor delaying the onset of breeding of red squirrels (Tamiasciurus hudsonicus) in British Columbia. Smith and Barkalow (1967) attributed early maturation and breeding of female gray squirrels to a large mast crop in the fall of 1965. Lint (1974) found reproductive attainment in the gray squirrel improved by supplemental feeding during two years of mast failure. These reports suggest that nutrition may augment productivity and bad weather delay breeding but neither factor is indicated as a major influence in the actual stimulation of seasonal breeding activity.

It is apparent that the need for more information regarding seasonal breeding exists. Mossman (1966:468) states that in order for sound generalizations to be made regarding reproductive function, "morphological studies must continue with and be ahead of physiological research.".

General Morphology of the Mammalian Ovary

Morphologically the ovaries of most mammals are quite similar. Variations in the degree of development of certain structures are, of course, present. Brambell (1956), Harrison (1962), Perry (1971), and Mossman and Duke (1973) provide general descriptions of the mammalian ovary from which the following has been condensed except where otherwise noted.

The surface of the ovary is usually covered by a cuboidal or low columnar epithelium called the germinal epithelium. This tissue often penetrates a subadjacent fibrous tunica albuginea in larger

species. The interior of the ovarian mass is frequently described as consisting of two zones, the medulla and the cortex. The cortex, or outer zone, contains the ova (primary oocytes), follicles, and luteal glands. The medulla is considered a zone of lymph and blood vessel proliferation. These designations are by no means distinct, as medullary follicles and oocytes are frequently encountered.

Probably the most prominent features are the follicles. Normal ovarian follicles can be placed into four classifications (Mossman and Duke 1973). The classifications and their corresponding characteristics are listed below:

1) Primordial - ova surrounded by a single layer of simple squamos epithelium
2) Primary - ova surrounded by a single layer of low columnar epithelium
3) Secondary - ova surrounded by a stratified cuboidal epithelium, the granulosa
4) Vesicular - mature follicles with intercullar fluid filled spaces which coalesce into a single large cavity, the antrum

Vesicular follicles are often called tertiary, antrum, or Graafian follicles. These follicles consist of three major cell zones, the theca externa, the theca interna, and the granulosa. The theca externa consists of compressed stromal cells adjacent to the theca interna, a distinct glandular area (Perry 1971). The theca interna is a concentric sheath of 3-4 layers of vacuolated, polygonal

cells. In all mammalian species studied by Mossman and Duke (1973) the maximuum glandular development of the theca interna is at estrus. Due to its function, this strata is best considered the thecal gland (Mossman and Duke 1973). Separating the theca interna and granulosa cells is the thin membrana propria. The granulosa cells, a cuboidal epithelium with a granular avascular cytoplasm, make up the major portion of secondary follicles in the form of a stratified core. In the tertiary follicle the granulosa is restricted to concentric rings around the antrum.

The ovum is contained in a projection of the granulosa called the cumulus oophorus. Adjacent to the ovum are radially arranged granulosa cells, the corona radiata. As early as the primary follicle stage, a homogenous mucopolysaccharide forms a layer encapsulating the ovum called the zona pellucida. The zona pellucida is pentrated by processes of the oocyte and the cells of the corona radiata.

Another prominent feature of the mammalian ovary is the corpus luteum, or more appropriately, the luteal gland (Mossman and Duke 1973). Following ovulation the membrana propria breaks down followed by a vascularization of the granulosa (Richardson 1967). These large polyhedral cells are formed in most species from both thecal and granulosa cells but may be derived from just the granulosa in some species (Blanchette 1966, Perry 1971). The luteal gland is richly supplied with blood and lymph capillaries and is usually larger when fully grown than the follicle from which it is derived. Frequently the luteal cells project through the ovulatory opening (the ovulatory

stigma) and form a node clearly visible on the ovarian surface. In species having a menstrual cycle rather than an estrous cycle, the luteal gland regresses rapidly if fertilization does not take place. During pregnancy its duration is longer, but it may or may not persist for the entire gestation.

The regressing luteal gland is characterized by smaller cells of reduced cytoplasmic and nuclear volume. There is more connective tissue interspersed among the luteal cells and a lumen filled with collagen fibers is evident (Hammond 1952). Eventually all that remains of the luteal gland is a dwindling scar of connective tissue called a corpus albicans. This structure tends to sink slowly into the medullary area.

Differentiated interstitial tissue (luteinized stroma) is usually present in most mammalian ovaries. Its origin is the theca internae of degenerate (atretic) follicles, and like luteal cells, this tissue consists of large polyhedral cells vacuolated with lipid droplets. It becomes particularly well developed during estrus and pregnancy. This well-differentiated interstitial tissue becomes so extensive in some species such as bats (Myotis spp.) that it is indistinguishable from luteal glands during pregnancy (Harrison 1962, Perry 1971). Certain rodents, including the sciurids, have maximum interstitial cell development at proestrus and estrus (Harrison 1962).

Morphology of the Rodent Ovary

Detailed histological studies of wild rodent ovaries other than the gray squirrel have been made for species such as voles (Microtus

spp., Greenwald 1956), nutria (Myocastor coypus, Stanley and Hilleman 1960, Rowland and Heap 1966), porcupines (Erithizon dorsatum, Mossman and Judas 1949) and red squirrels (T. hudsonicus, Millar 1970, Mossman and Duke 1973).

The ovary of infantile rodents is characterized by a thick cortex containing many naked ova and primary oocytes. Medullary cords of epithelial tissue containing ova more advanced than those of the cortex are conspicuous. The prepubertal ovary shows the formation of interstitial tissue derived from small atretic tertiary follicles (Mossman 1966). In the prepubertal red squirrel, primordial and primary follicles can be found in two distinct zones (Mossman and Duke 1973). Prepubertal gray squirrels appear to have successive batches of follicles grow and become atretic until the first proestrus. The maximum size of their follicles is about 600 μm (Deanesly and Parkes 1933). These prepubertal ovaries can be distinguished from mature ovaries in that they do not contain the vestiges of earlier ovulatory periods (Mossman and Duke 1973).

In the pubertal ovary of most rodents there is usually a complete disappearance of medullary follicles (Mossman 1966). Primordial and primary follicles are scarce. As the red squirrel approaches estrus, there are numerous corpora atretica and more extensive development of the interstitial tissue (Mossman and Duke 1973). Compared to most mammals the theca interna of sciurids is relatively thin, while interstitial tissue is more plentiful (Mossman and Duke 1973). Deanesly and Parkes (1933) note that in adult gray squirrels there is

little interstitial tissue to be found and do not mention its extent during estrus and pregnancy.

In regard to numbers and development of tertiary follicles, there is considerable variation during anestrus in adult gray squirrels. As in prepubertal animals, the maximum diameter of tertiary follicles is about 600μm in anestrus adults (Deanesly and Parkes 1933). Parous red squirrels have follicles ranging from 500μm to 700μm during anestrus (Mossman and Duke 1973). Brauer and Dusing (1961) note the presence of tertiary follicles in the ovaries of 11 of 13 adult gray squirrels collected over a period of one year. There were no tertiary follicles in one squirrel collected in December or in one squirrel collected in January. They found large numbers of tertiary follicles in squirrels taken during September, November, February and March. On the other hand, the blacktail prairie dog (Cynomys ludovicianus) is a sciurid that develops tertiary follicles in large numbers only during the breeding season (Foreman 1962). The mountain beaver (Aplodontia rufa) also has almost no follicular development in anestrus as well (Pfeiffer 1958).

The development of a group of follicles immediately before ovulation in the gray squirrel is accompanied by extensive degeneration of remaining smaller follicles. This is reflected by an overall decrease in numbers of follicles. One specimen thought to be in estrus had only 4 mature follicles while anestrus animals had up to 15 tertiary follicles in the ovary. Mature follicles close to ovulation had about twice the diameter of those of anestrus squirrels,

the average diameter of four mature follicles being 1010 µm (Deanesly and Parkes 1933). In the red squirrel (T. Hudsonicus), the largest follicle may exceed a diameter of 1 mm at estrus as well. Loosening and separation of the cumulus cells were typical of all fully ripe follicles. As in the gray squirrel, the ripening of several large follicles is accompanied by degeneration of many other follicles. These ripe follicles are so distended that they occupy the medulla of the ovary as well (Mossman and Duke 1973).

During early pregnancy the dominant features of the rodent ovary are the developing luteal glands and fully differentiated interstitial gland tissue. The luteal gland may have a peripheral theca-like zone of cells (Mossman 1966). In one gray squirrel Deanesly and Parkes (1933) noted luteinization of the granulosa even though the follicle failed to ovulate. Usually by late pregnancy there is a decline in size and quantity of luteal cells and interstitial cells. If the species has a postpartum estrus, tertiary follicles are present and more interstitial tissue becomes apparent. The ovary of such species at this time is similar to the proestrus ovary of long cycle species except for the presence of the luteal glands (Mossman 1966). Deanesly and Parkes (1933) state that the regression of the luteal glands begins in mid-pregnancy and final disappearance is during lactation. Since they found some pregnant animals with large numbers of tertiary follicles, they suggest that follicular growth continues during pregnancy but is not accompanied by normal atresia. They describe the gray squirrel as failing to have a postpartum estrus.

The fate of luteal glands in rodents is not always that generally described by Mossman (1966). In the red squirrel (T. hudsonicus), the luteal glands of the spring pregnancy persist during the second pregnancy and may even last into the second lactation. They are recognized by their larger size and larger vacuolated cells (Mossman and Duke 1973). In the mountain beaver, regression of the luteal glands begins after parturition (Pfeiffer 1958).

In general, species without a postpartum estrus have persistant luteal glands during lactation (Mossman 1966). There is a scarcity of follicles other than primary and primordial, as noted in the red squirrel (T. hudsonicus). Also of significance is the complete disappearance of interstial cells that have changed to the stromal type (Mossman 1966). Deanesly and Parkes (1933) observed that follicles during lactation are usually no larger than 600μm, with little variation in size as lactation passes into anestrus in the gray squirrel.

Growth and Atresia of Follicles

Two complementary processes occurring in the ovary are follicular growth and atresia. Of thousands of primary oocytes only a few are destined to be ovulated, the rest will degenerate at some stage. Follicular growth consists primarily of two phases (Harrison 1962). The first phase is one in which the oocyte grows rapidly while the follicle is still in the primary stage. When the follicle reaches a diameter of 250 to 350μm an antrum usually forms (Deanesly and Parkes 1933). In the second phase of growth, the follicle grows rapidly and

ovum changes little if at all. The final size of the follicle is directly related to body size of the species (Harrison 1962). During the second phase of growth, cytoplasmic vesicles discharge the liquor folliculi into the intercellular spaces. The droplets eventually coalesce and fill the antrum. Another important event during this preovulatory growth is the loosening of cells forming the cumulus oophorus and its eventual separation from the underlying granulosa (Greenblatt 1966).

It is not known what causes so many of these follicles (and oocytes) to become atretic. There is no way to distinguish those which survive from those that will eventually degenerate (Perry 1971). Atretic oocytes are difficult to identify in the early stages, but relatively easy in medium and large follicles. The nuclear membrane of the oocyte usually breaks down followed by nuclear fragmentation. Granulosa cells become detached and float in the antrum. The liquor folliculi becomes cloudy and gelatinous. Hypertrophy of the theca interna forming an interstial node occurs as well (Ingram 1962), Mossman and Duke 1973). This hypertrophied theca interna is quite important in its endocrine function (Perry 1971). Factors which can affect the rate of atresia include age, reproductive state, nutrition, hypophysectomy, ovarian grafting, x-irradiation and treatment with exogenous hormones (Ingram 1962). Ingram (1962) suggests three functions of follicular atresia:

1) The amount of hormones supplied by ovulatory follicles may be insufficient to prime the

reproductive tract. It is supplemented by hormones secreted by interstitial cells formed from the theca internae of atretic follicles.

2) Atresia is a selective process where only the fittest survive.

3) Atresia causes a reduction of oocyte supply in order to influence fertility in relation to age.

Endocrine Role of the Ovary

Like many endocrine glands, the ovaries are responsive to levels of hormones released by the anterior pituitary. It is generally understood that the protein hormones FSH and LH are responsible for follicular growth and ovulation (with subsequent luteinization) respectively (Fevold et al. 1931). Actually the stages of the ovarian cycle are initiated by changes in the FSH/LH ratio rather than by the replacement of one by the other (Perry 1971).

Estrogen production by the ovary is at its peak during preoestrus and estrus. Since the thecal gland and interstitial tissues show maximum development during these periods, it seems likely the two phenomena are related. A number of histochemical studies have borne out that they are indeed the primary zones of estrogen production (Rennels 1951, Harrison 1962, Goldenberg et al. 1973).

It is believed the rise in estrogen level stimulates the release of LH while simultaneously acting as a negative feedback on the anterior

pituitary inhibiting FSH secretion (Greenblatt 1966). Although estrogen administration had no effect on gonadotropin levels in ovarectomized rats (Richardson 1967), a proper level will release LH after a single injection early in the estrus cycle of ewes (Howland et al. 1968). In general, during ovarian maturation leading to ovulation, estrogen may have the following effects (Richardson 1967):

1) Intraovarian effect on the granulosa leading to follicular development
2) Long term, low level, inhibitory effect on gonadotropin release
3) Short term, high level, stimulatory effect on gonadotropin release
4) Priming effect on tissues of reproductive tract in preparation for breeding

Dixon and Marshall (1931) were first to determine that the luteal glands of pregnancy inhibit action of the anterior pituitary. The principal hormone involved in this inhibitory activity is progesterone, formed by the luteal cells (Perry 1971). High levels of progesterone acting on the pituitary actively block gonadotropin release and thus allow gestation to proceed. When progesterone levels decline in late pregnancy the uterus becomes responsive to oxytocin (Perry 1971).

The entire role of progesterone is far more complex. It has been shown that ovulation cannot occur in the absence of it. It appears

that progesterone participates in the stimulation of the high rate of LH release leading to ovulation (Richardson 1967).

In most mammals, the luteal gland inhibits the maturation of follicles via its inhibition of gonadotropin secretion. The active secretion of progesterone ends before the morphological changes are evident. The involution of the luteal cells is due to the release of enzymes from cytoplasmic inclusions called lysosomes. There is evidence that a uterine secretion affects the life of the luteal gland since hysterectomy extends their duration in some species (Perry 1971). Blatchley and Donovan (1971) have demonstrated that prostaglandin F-2-α causes luteolysis in guinea pigs. This prostaglandin has been detected in the uterine lining and the utero-ovarian arteries of other mammals.

In rodents which lack a postpartum estrus, the luteal glands which persist during lactation (Mossman 1966) are probably non-functional. Thus the luteal gland is not the primary factor which delays the postpartum estrus. However, suckling appears to block the release of FSH from the pituitary with consequent reduction of follicular development in swine (Lauderdale et al. 1965) and may affect other species similarly.

Effects of Exogenous Hormones

For 40 years it has been possible to induce ovulation in laboratory animals using pituitary extracts (Richardson 1967). Attempts to do so with captive wild animals have been done more recently as

reviewed by Mellace (1973). Results among and within studies have been typically quite variable due to the complexities of effects such as age, season, treatment schedules, dosage levels, stress, and variations in composition of hormone extractions.

For example, the acid-acetone method of Steelman et al. (1953) for fractionation of FSH from the pituitary of domestic animals yields an alcohol fraction of FSH contaminated with LH. This procedure, adopted by most commercial suppliers, has been of some advantage to researchers since it has been shown that highly purified FSH does not stimulate follicular growth. Lostroh and Johnson (1966) found that using purified FSH or LH produced no follicular changes in rats. However, as little as 0.05 μg of LH with 3.00 μg FSH caused an increase in follicular growth. They concluded that LH together with FSH is essential for follicular growth and estrogen secretion in the rat. Thus, lack of control of LH content of commercial preparations probably is an important contributor to variation in results frequently encountered.

Luteinization of the granulosa without ovulation is especially frequent in animals treated with pituitary hormones. Hypertrophy of the theca interna may also occur (Ingram 1962). Hammond (1952) noted the occurrence of many luteal glands with retained ova in the ovaries of mink treated with PMS and PU (pregnancy urine, an impure form of chorionic gonadotropin). Lostroh and Johnson (1966) observed similar results in rats receiving 3.00 μg FSH and 1.00 μg LH concomitantly for several days. In juvenile mice, three days of FSH treatment causes

the interstitial cells to luteinize (Ben-or 1963). Such results may be the function of dosage level as excess FSH may lead to cystic follicles and excess LH may cause luteal glands with retained ova (Ingram 1962). It may also be related to the timing of LH administration. In rats, the best results were obtained by injecting LH on the fourth day of treatment whereas if the ovulatory dose was one day later large numbers of ova were entrapped in luteinizing follicles (Lostroh and Johnson 1966).

The effects of exogenous treatments are also mediated by endogenous hormone sources. The capacity of the ovary to respond to FSH stimulation develops gradually with age, in the very young it is entirely lacking (Ben-or 1963). Experiments by Goldenberg (1973) show that ovarian responsiveness is dependent on estrogen produced by the theca interna, which in laboratory rats does not develop until the age of 6-8 days. The direct action of endogenous sources of estrogen appears to be the prevention of follicular degeneration (Ingram 1962). Therefore, exogenous sources of estrogen and androgen inhibit the formation of interstitial tissue (Rennels 1951).

The ovulatory response to exogenous hormones may be related to the spontaneous release of LH in some species (Seth and Prasad 1967). Also of interest is the fact that if the purified substance is utilized, exogenous FSH will work as well as LH as an ovulatory stimulus (Lostroh and Johnson 1966).

In general, when exogenous application of gonadotropin succeeds at all in the induction of ovulation it usually leads to super-

ovulation (Richardson 1967) such as experienced by Seth and Prasad (1967) and Murphy (1973). Failures are characterized by luteal glands with entrapped ova and/or large luteinized cysts.

MATERIALS AND METHODS

Wild Squirrels

A total of 109 female gray squirrels (67 adults and 42 subadults) was collected over a period of one year between December, 1966 and November, 1967 in the vicinity of Blacksburg, Virginia[1]. Ovarian and uterine weights (empty) for each specimen were recorded as well as date of collection, number of placental scars, number of fetuses and age. Aging was done by a combination of pelage characteristics (Sharp 1958), body weight and general appearance (Barrier and Barkalow 1967, Taber 1969:381). The animal's reproductive state with regard to pregnancy, lactation, and perforation of the vaginal orifice was noted as well. Each female was classified as one of the following:

a) vaginal orifice closed (no fetuses, not lactating)

b) vaginal orifice open (no fetuses, not lactating)

c) pregnant (fetuses present)

d) lactating

One hundred and ninety-four ovaries removed from these squirrels were fixed in Bouin's solution and embedded in paraffin. All were sectioned serially using a Spencer 820 microtome at 10 μm thickness. Every tenth section was placed on a 50 mm x 75 mm slide coated with Mayer albumin fixative. Prior to staining the sections were hydrated

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[1]Collected by J. O. Newell, Graduate Research Assistant, V.P.I.&S.U., (deceased).

through two changes of xylene, two changes of absolute ethyl alcohol, one change of 95% ethyl alcohol, one change of tap water and one change of distilled water. They were then stained with Harris-Lillie hematoxylin and counterstained with 0.5% alcoholic eosin. Dehydration through three changes of 95% ethyl alcohol, two changes of absolute alcohol, and three changes of xylene was accomplished utilizing a Fisher Tissuematon (Fisher Scientific, Pittsburgh, Pa.). After staining, a cover glass was mounted permanently on each slide using permount.

Microscopic measurements of tertiary follicles and luteal glands were made using a whipple ocular grid at a magnification of 40X. Follicles considered tertiary were those containing a completely formed antrum. Also follicles with scattered areas of follicular fluid within the granulosa were considered tertiary. These follicles corresponded to large follicles in categories 6 to 8 as described by Pederson and Peters (1968) for the mouse ovary. Since the gray squirrel ovary contains numerous tertiary follicles of variable size and shape, an average diameter size class system was employed. At 40X it was possible to calibrate the ocular grid to the nearest 36μm. Thus thirty-two size classes were established covering a range of approximately 300 to 1420μm average diameter (Table 1). Average diameter class of each follicle was estimated using the average of two measurements taken at right angles. These were the largest diameter and the largest diameter perpendicular to it. The inner edge of the theca interna was considered the perimeter of tertiary follicles. The following data for each

Table 1. Arbitrary diameter size classes established for tertiary follicles in the present study.

Follicle size class	Class limits (microns)	Size class average diameter (microns)
1	289-324	306
2	325-360	342
3	361-396	378
4	397-432	414
5	433-468	450
6	467-504	486
7	505-540	522
8	541-576	558
9	577-612	594
10	613-648	630
11	649-684	666
12	685-720	702
13	721-756	738
14	757-792	774
15	793-828	810
16	829-864	846
17	865-900	882
18	901-936	918
19	937-972	954
20	973-1008	990
21	1009-1044	1026
22	1045-1080	1062
23	1081-1116	1098
24	1117-1152	1134
25	1153-1188	1170
26	1189-1224	1206
27	1225-1260	1242
28	1261-1296	1278
29	1297-1332	1314
30	1333-1368	1350
31	1369-1404	1386
32	1405-1440	1422

ovary were recorded:

a) average diameter class of each tertiary follicle

b) number of luteal glands

From these measures additional variables were created combining the observations of both ovaries of each animal. Since both ovaries were not available for 19 of the squirrels originally collected, only 90 animals (53 adults and 37 subadults) were represented by these combined data. The following variables were subjected to statistical analysis:

a) number of tertiary follicles

b) sum of diameters of all tertiary follicles

c) diameter of the largest tertiary follicle

d) average diameter of the four largest tertiary follicles

Within age classes, means and standard errors were computed for reproductive states and for months of all variables. Homogeneity of variance was tested using the F-Max test (Sokal and Rohlf 1969). Where necessary, logarithmic transformation was utilized in order to stabilize the variance and the F-Max test was repeated on transformed means. If appropriate, a factorial analysis of variance using a least squares regression procedure (Barr and Goodnight 1971) was utilized to determine significance of effects due to age and season. Also a factorial analysis of variance was performed to determine significance of effects due to age and reproductive state. One-way analysis of variance was also used within age classes to test differences among reproductive states. Duncan's New Multiple Range Test was applied to

means showing significant differences in the analyses of reproductive states (Steel and Torrie 1960).

Comparisons were made among luteal gland, fetal, and placental scar counts in order to calculate the extent of prenatal mortality. Comparison of macroscopic and microscopic counts of luteal glands were made in order to determine the accuracy of the former.

Captive Squirrels

During the period of October 1973 to April 1974, adult female gray squirrels were trapped from woodlots located on the Virginia Polytechnic Institute and State University campus and farm. Upon capture the animals were marked with ear tags and weighed. Aging was done by examination of pelage, body condition, and mammae (Sharp 1958, Taber 1963). Each squirrel was placed in an individual indoor laboratory cage. The cages measured 56 cm x 34 cm x 26 cm, and each contained a small nest box. The room housing the squirrels was maintained at approximately 22 degrees Centigrade and on a 14:10 light: dark ratio. Water and feed were supplied ad libitum. The feed, a commercial food designed for rats and mice (Wayne Lab-Blox F6, Allied Mills, Inc.,), contained approximately 24 percent crude protein, 6 percent crude fat and 5 percent crude fiber.

Two experiments were performed on a total of 21 squirrels. Three different hormone preparations were administered in the course of these experiments. These were preparations of follicle stimulating hormone (NIH-FSH-S-6, National Institute of Health, Betheseda, Maryland; and FSH-P, Armour-Baldwin Laboratories, Omaha, Nebraska)

and of human chorionic gonadotropin (HCG, Nutritional Biochemical Corporation, Cleveland, Ohio). The hormones were received in a lyophilized state and kept under refrigeration until diluted with sterile saline (0.9 percent NaCl) immediately prior to use.

Nest boxes were removed from the cages during each experiment in order to facilitate catching the squirrels. A rectangular burlap net was used to capture each animal immediately prior to injection. While in the burlap net, the squirrel was held firmly behind the head with heavy gloves. The net was then pulled back exposing the animal. No anesthetic was necessary while giving subcutaneous or intravenous injections. All injections were given between 11 and 12 a.m.

At the completion of each experiment the squirrels were sacrificed, weighed and the reproductive tracts were removed. Adipose and connective tissues were carefully removed from the tracts. The cervix was excised at the constriction that marks the utero-cervical junction. The ovaries were removed from the ovarian bursa and oviducts were cut off at the junction with the uterine cornua. The following measurements were made using a precision balance and vernier caliper and recorded:

a) the length, width and depth of each pair of ovaries (These values were then summed and this sum was recorded and analyzed as the ovarian index.)

b) the width of the uterine cornua at the utero-tubal junction (these values were then summed and

this sum was recorded and analyzed as the uterine index.)

c) paired ovarian weight

d) uterine weight

The ovaries were then fixed in Bouin's solution, embedded in paraffin, sectioned, stained and measurements made as described for the wild squirrels.

Experiment 1

Experiment 1 was designed for two purposes. The first objective was to determine if luteal glands would form after the application of FSH alone as observed in one squirrel treated by Mellace (1973). The second objective was to determine if any difference in response would occur between animals receiving the HCG injection subcutaneously and those receiving it intravenously.

The experiment was begun in April, 1974. Hormones administered were FSH-P and HCG. Twelve adult female gray squirrels were assigned randomly to 4 groups of 3 squirrels each as shown in Table 2. Group 1 received 0.5 ml of 0.9 percent saline subcutaneously per day on days 1, 2, 3, 4, 5, 6, 7 and 13. Group 2 received 2 mg FSH in 0.5 ml saline per day on days 1, 2, 3, 4, 5, 6, 7 and 0.5 ml saline subcutaneously on day 13. Group 3 was given 2 mg FSH in 0.5 ml saline per day on days 1, 2, 3, 4, 5, 6, 7 and 400 IU HCG in 0.5 ml saline subcutaneously on day 13. Group 4 received the same injection regime as Group 3 except that the HCG injection was made intravenously into the great saphenous vein. All subcutaneous injections were made in the dorsal

Table 2. Schedule of exogenous hormone treatment of captive gray squirrels in Experiment 1.

Group	Number of animals	Day of sacrifice	Treatment Saline	FSH	HCG
1	3	15	0.5 ml of 0.9% NaCl per day. Days 1,2,3,4,5, 6,7 and 13. Subcutaneously.	--	--
2	3	15	0.5 ml saline on day 13. Subcutaneously.	2.0 mg in 0.5 ml saline per day. Days 1, 2,3,4,5,6 and 7. Subcutaneously.	--
3	3	15	--	2.0 mg in 0.5 ml saline per day. Days 1, 2,3,4,5,6 and 7. Subcutaneously.	400 IU in 0.5 ml saline on day 13. Subcutaneously.
4	3	15	--	2.0 mg in 0.5 ml saline per day. Days 1, 2,3,4,5,6 and 7. Subcutaneously.	400 IU in 0.5 ml saline on day 13. Intravenously.

region. On the fifteenth day the squirrels were sacrificed and measurements were made as described above.

Experiment 2

In July, 1974 a second experiment was conducted with the purpose of comparing the hormonal treatment methods of Williams et al. (unpublished) to those developed by Mellace (1973). Williams et al. (unpublished) reported the formation of luteal glands in gray squirrels following the application of a lower dosage of FSH and an earlier injection of HCG than utilized by Mellace (1973). The injection regime used in Groups 1 and 2 of this experiment was patterned after the methods of Williams et al. (unpublished) and in Group 3 the regime was patterned after the methods of Mellace (1973). Also the experiment was designed to compare the effects of two different FSH preparations, FSH-P and NIH-FSH-S-6.

Nine adult female gray squirrels were randomly assigned to 3 groups of 3 animals each (Table 3). All injections were given subcutaneously. Group 1 received 200 μg NIH-FSH in 0.1 ml saline per day on days 1, 2, 3, 4, 5, 6, 7 and 200 IU HCG in 0.4 ml saline on day 5. The squirrels in this group were sacrificed on day 8. Group 2 was treated in the same fashion except that the FSH was the Armour-Baldwin preparation (FSH-P). Group 3 received 2 mg FSH-P in 0.5 ml saline per day on days 1, 2, 3, 4, 5, 6, 7 and 200 IU HCG in 0.4 ml saline on day 13. Animals in Group 3 were sacrificed on day 15. Although unknown at the time, squirrel #4006 was pregnant when captured and was taken off the experiment on the third day following the birth of 2 young.

Table 3. Schedule of exogenous hormone treatment of captive gray squirrels in Experiment 2.

Group	Number of animals	Day of sacrifice	Treatment	
			FSH	HCG
1	3	8	200 μg in 0.1 ml saline per day. Days 1,2,3,4,5,6 and 7. NIH-FSH. Subcutaneously.	200 IU in 0.4 ml saline on day 5. Subcutaneously.
2	3	8	200 μg in 0.1 ml saline per day. Days 1,2,3,4,5,6, and 7. FSH-P, A.-B. Lab. Subcutaneously.	200 IU in 0.4 ml saline on day 5. Subcutaneously.
3	3	15	2.0 mg in 0.5 ml saline per day. Days 1,2,3, 4,5,6 and 7. FSH-P, A.-B. Lab. Subcutaneously.	200 IU in 0.4 ml saline on day 13. Subcutaneously.

Measurements of ovaries and uteri were made as described previously. In addition, oviducts were fixed in Bouin's solution, embedded in paraffin, sectioned and stained. They were then examined microscopically to determine if ova were present.

For both experiments, means and standard errors were computed by group for all variables. Homogeneity of variance was tested using the F-Max test (Sokal and Rohlf 1969). One-way analysis of variance was utilized to determine significance of effect due to treatment. Duncan's New Multiple Range Test was applied to means showing significant differences in the above analyses (Steel and Torrie 1960). Data from squirrel #4038 was not included in the analysis of Experiment 2 since it was found that it had atypical ovaries lacking primary oocytes and follicles (Appendix Fig. VIII).

RESULTS AND DISCUSSION

Wild Squirrels

Analyses by Reproductive State

The effect of reproductive state on the various ovarian and uterine characteristics is presented first because it will facilitate the discussion of seasonal changes. Differences in paired ovarian weights and uterine weights among reproductive states were highly significant ($P<0.01$) as shown in Table 4. There were no significant differences between ages nor were there significant interactions. Duncan's New Multiple Range Test showed that pregnant adults had significantly higher ($P<0.05$) mean paired ovarian weights than animals lactating or those with closed vaginas. The means of uterine weight in the four reproductive states were all significantly different (Table 5). Table 5 shows that mean paired ovarian weights and mean uterine weights of pregnant adults (51.8 mg and 4298.3 mg respectively) were followed by those with open vaginas (38.1 mg and 1875.7 mg respectively), those lactating (29.9 mg and 584.0 mg respectively) and those with closed vaginas (24.2 mg and 330.9 mg respectively). Essentially similar results were observed in subadults (Table 6).

The elevated ovarian and uterine weights of pregnant animals can be explained by the presence of luteal glands on the ovaries and the proliferation of uterine tissues during pregnancy. Ovarian and uterine weights of animals with open vaginas were higher than those of animals with closed vaginas. It is probable that the former were near estrus. In squirrels with open vaginal orifices, the

Table 4. Mean squares for age by reproductive status analysis of ovarian and uterine weight of gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967.

Source of variation	Degrees of freedom	Log_{10} paired ovarian weight ($\times 10^{-2}$)	Log_{10} uterine weight ($\times 10^{-2}$)
Age	1	7.6	1.6
Reproductive status	3	20.0**	401.6**
Age x reproductive status	3	3.2	21.0
Error	100	2.4	10.8

**$P < 0.01$

Table 5. Means by reproductive status for ovarian and uterine weight of adult gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967 (means ± standard error).

Reproductive status	Number of animals	Paired ovarian weight (mg)	Uterine weight (mg)
Vaginal orifice closed (no fetuses)	20	24.2 ± 2.2^{b}	330.9 ± 61.2^{a}
Vaginal orifice open (no fetuses)	5	38.1 ± 4.5^{ab}	1875.7 ± 354.9^{b}
Pregnant	15	51.8 ± 6.9^{a}	4298.3 ± 796.4^{c}
Lactating	26	29.9 ± 1.8^{b}	584.0 ± 159.0^{d}

^{a,b,c,d}Means in a column bearing the same superscript are not significantly different ($P<0.05$).

Table 6. Means by reproductive status for ovarian and uterine weight of subadult gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967 (means ± standard error).

Reproductive status	Number of animals	Paired ovarian weight (mg)	Uterine weight (mg)
Vaginal orifice closed (no fetuses)	33	24.0 ± 1.6[a]	211.2 ± 30.1[a]
Vaginal orifice open (no fetuses)	2	32.9 ± 0.5[a]	2077.5 ± 265.3[b]
Pregnant	2	36.9 ± 0.3[a]	2144.5 ± 201.9[b]
Lactating	5	20.2 ± 3.0[a]	961.9 ± 401.1[b]

[a,b]Means in a column bearing the same superscript are not significantly different ($P<0.05$).

ovaries had large, fluid filled follicles or recently formed luteal glands and the uterus, under the influence of estrogen produced by these follicles, was also in a more developed state. The higher mean paired ovarian weight of lactating animals compared to those with closed vaginas was probably due to the presence of luteal glands on several ovaries of the former.

Differences among reproductive states in the number of tertiary follicles and the sum of the follicular diameters were significant ($P<0.05$) as shown in Table 7. There were no significant differences between ages. Also there were no significant interactions. Duncan's New Multiple Range Test showed that pregnant adults had significantly ($P<0.05$) more tertiary follicles than all other reproductive states. Also the sum of the follicular diameters in pregnant adults was significantly higher ($P<0.05$) than in all other reproductive states. The number of tertiary follicles (29.7) and the sum of the follicular diameters (15,954.7 μm) was highest in pregnant adults, next highest in lactating adults (18.1 and 9742.5 μm respectively), next highest in adults with closed vaginas (14.4 and 7792.5 μm respectively) and lowest in adults with open vaginas (8.7 and 5678.9 μm respectively) (Table 8).

The reduction in the number of tertiary follicles of gray squirrels near estrus was consistent with the findings of Deanesly and Parkes (1933). Mossman and Duke (1973) found a similar situation for the red squirrel (T. hudsonicus). Increased atresia would account for the decline in numbers of follicles prior to estrus. An abundance of

Table 7. Mean squares for age by reproductive status analysis of follicular measurements of gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967.

Source of variation	Degrees of freedom	No. of tertiary follicles	Sum of follicular diameters	Diameter largest follicle ($\times 10^4$)	Log_{10} average diameter four largest follicles[a] ($\times 10^{-3}$)
Age	1	0.01	219.8	2.6	8.2
Reproductive status	3	321.52*	9.6×10^7*	7.6	14.4*
Age x reproductive status	3	50.82	9.9×10^6	4.3	6.2
Error	81	124.87	3.4×10^7	6.1	3.7

[a]Error mean square has 71 degrees of freedom because squirrels with no tertiary follicles were deleted.

*$P < 0.05$

follicles during pregnancy was also observed by Deanesly and Parkes (1933) who postulated that there was decreased atresia of smaller follicles at this time. The mechanisms involved are obscure, but are probably related to those causing increases in average follicular diameters during pregnancy, as described later.

Follicular numbers and sum of follicular diameters of lactating animals appeared to approximate those of squirrels in anestrus, although in each age class lactating squirrels had numerically higher means (Tables 8 and 9). The close correspondence between follicular numbers and sum of follicular diameters suggest that the latter is probably a function of the former.

There were no significant differences among reproductive states or between ages for the average diameter of the largest follicle (Table 7). Means for adults and subadults are shown in Tables 8 and 9 respectively. One of the adults with a perforate vaginal orifice had a total of four large follicles, the largest of which had an average diameter of 1240 μm. This exceeded the maximum follicular diameter of 1100 m reported by Deanesly and Parkes (1933) for an estrous adult gray squirrel.

Analysis of variance of the average diameter of the four largest follicles of ovaries that containtained tertiary follicles (some observations had none and the transformation could not be made) showed that significant differences ($P<0.05$) existed among reproductive states but not between ages (Table 7). Duncan's New Multiple Range Test for the overall means of both ages together showed that squirrels with

Table 8. Means by reproductive status for follicular measurements of adult gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967 (means ± standard error).

Reproductive status	Number of animals	No. of tertiary follicles	Sum of follicular diameters (μm)	Diameter largest follicle (μm)	Average diameter four largest follicles (μm)
Vaginal orifice closed (no fetuses)	13	14.4 ± 3.3[a]	7792.9 ± 1764.2[a]	583.8 ± 75.6[a]	527.6 ± 68.4
Vaginal orifice open (no fetuses)	4	8.7 ± 5.0[a]	5678.9 ± 2700.2[a]	850.0 ± 293.2[a]	702.4 ± 241.0
Pregnant	11	29.7 ± 3.9[b]	15954.7 ± 1826.7[b]	769.1 ± 24.8[a]	714.1 ± 26.7
Lactating	24	18.1 ± 2.4[a]	9742.5 ± 1270.9[a]	613.3 ± 51.9[a]	573.4 ± 48.5

[a,b]Means in a column bearing the same superscript are not significantly different ($P<0.05$).

Table 9. Means by reproductive status for follicular measurements of subadult gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967 (means ± standard error).

Reproductive status	Number of animals	No. of tertiary follicles	Sum of follicular diameters (μm)	Diameter largest follicle (μm)	Average diameter four largest follicles (μm)
Vaginal orifice closed (no fetuses)	29	17.4 ± 1.9[a]	9263.8 ± 1013.4[a]	592.4 ± 40.6[a]	559.7 ± 38.3
Vaginal orifice open (no fetuses)	2	10.5 ± 9.5[a]	5810.3 ± 5359.4[a]	560.0 ± 110.0[a]	324.3 ± 324.2
Pregnant	2	22.5 ± 4.5[a]	12998.3 ± 1973.2[a]	770.0 ± 0.0[a]	729.5 ± 9.0
Lactating	4	20.5 ± 1.3[a]	11116.3 ± 1078.2[a]	682.5 ± 79.7[a]	659.7 ± 80.6

[a]Means in a column bearing the same superscript are not significantly different ($P<0.05$).

open vaginal orifices had a significantly higher ($P<0.05$) mean than all other reproductive states. Table 8 shows that the average diameter of the four largest follicles of adults with open vaginal orifices and pregnant adults (702.4 μm and 714.1 μm respectively) were greater than lactating adults or adults with closed vaginal orifices (573.4 μm and 527.6 μm respectively). The average diameter of the four largest follicles (729.5 μm) was highest in pregnant subadults, next highest in lactating subadults (659.7 μm), next highest in subadults with closed vaginas (559.7 μm) and lowest in subadults with open vaginas (324.3 μm) (Table 9).

The data of the present study on the diameter of the largest follicle and the average diameter of the four largest follicles of squirrels with a closed vaginal orifice confirm the observation of Deanesly and Parkes (1933) that in anestrus squirrels follicular size rarely exceeds a "resting" diameter of about 600 μm. The means for these two variables in pregnant squirrels in the present study also confirm the findings of the above workers in that follicular growth past the "resting" size does occur during pregnancy. This is comparable to findings in elk (Morrison 1960) and white-tailed deer (Kirkpatrick 1974) both of which show considerable follicular development during pregnancy. Possibly effects of gonadotropin stimulus during proestrus and estrus were still being demonstrated by these follicles during pregnancy. The generally high values of follicular diameters in animals with a perforate vaginal orifice reflects elevated gonadotropin stimulation of the follicles.

Seasonal Analyses

As shown in Table 10, two separate periods of pregnancy and lactation occurred in squirrels used in this study. Pregnancy frequencies were highest in February (3 of 9 pregnant) and July (4 of 4 pregnant) while lactation peaks occurred in May (6 of 10 lactating) and October (10 of 13 lactating).

Deanesly and Parkes (1933), Allen (1952), Redmond (1953), Uhlig (1955), Smith (1967), and Newell and Kirkpatrick (1968) have reported two distinct periods of parturition for the gray squirrel as well. The two unequal periods of anestrus and the lack of a postpartum estrus has been interpreted as evidence for a single polyestrous breeding season for this species (Deanesly and Parkes 1933). Thus, instead of true interrupted breeding, one long breeding season with two peaks of breeding intensity may exist. The biannual occurrence of such peaks could be due to the relatively long gestation and lactation periods which would allow only two litters (Sadlier 1969). In the gray squirrel gestation and lactation together amount to a period of more than 16 weeks (Uhlig 1955) which could result in such a pattern.

Due to lack of further information, it is only possible to extrapolate from findings of studies such as those of Muul (1969b), Hoffman and Kirkpatrick (1959), and Woitkewitsh (1945) to explain the regulation of the breeding season of the female gray squirrel. To do so is not unrealistic since it is plausible that sympatric "diestrual" species (S. carolinensis and G. volans) are subjected to similar environmental stimuli such as photoperiod. For the flying squirrel, annual decrease

Table 10. Reproductive data obtained by necropsy of female gray squirrels collected each month of the year in Blacksburg, Virginia, December 1966 - November 1967.

Month	No. of females examined	No. with closed vagina (no fetuses)	No. with open vagina (no fetuses)	No. pregnant	No. lactating	Mean no. placental scars[a] ±SE	Mean no. fetuses ±SE
Dec.	8	8	0	0	0	0	0
Jan.	6	4	2	0	0	0	0
Feb.	9	5	1	3	0	0	3.0 ± 0.6
Mar.	10	5	0	1	4	2.2 ± 0.2(4)	2.0 ± 0.0
Apr.	10	6	0	0	4	3.2 ± 0.5(4)	0
May	10	4	0	0	6	2.2 ± 0.4(8)	0
June	13	10	3	0	0	2.0 ± 0.0(2)	0
July	4	0	0	4	0	0	3.8 ± 0.3
Aug.	11	3	1	5	2	3.0 ± 0.3(5)	2.6 ± 0.4
Sept.	11	2	0	3	6	3.0 ± 0.4(6)	4.0 ± 0.6
Oct.	13	3	0	0	10	2.4 ± 0.2(12)	0
Nov.	4	4	0	0	0	2.5 ± 0.5(2)	0

[a]Number of females with placental scars is in parentheses.

in photoperiod may trigger reproduction and annual increase in photoperiod may influence regression of the gonads (Muul 1969a). However, this theory does not adequately explain the regulation of seasonal breeding in the female gray squirrel. For example, Table 10 shows that a gray squirrel was near estrus in August which is contrary to what would be expected according to Muul's (1969a) explanation. Until more definitive studies are made, the identity of the primary factor(s) responsible for regulation of seasonal breeding in the gray squirrel will remain unknown.

Significant monthly differences in paired ovarian weights ($P<0.05$) and uterine weights ($P<0.01$) occurred as shown in Table 11. Significant differences ($P<0.01$) between ages were present. A significant age by month interaction in paired ovarian weights ($P<0.01$) and uterine weights ($P<0.05$) also was found. In adults, maximum mean paired ovarian weights (Table 12 and Fig. 1) occurred in February and July whereas maximum mean uterine weights (Table 12 and Fig. 2) occurred in February and August. Subadults showed peak ovarian and uterine weights in the summer months.

In general, paired ovarian weights and uterine weights reflected the occurrence of two pregnancy periods. The lack of a distinct winter peak in ovarian and uterine weights for subadults was probably because few summer born subadults breed the following winter whereas both spring born and summer born of the previous year are in breeding condition during the summer (Brown and Yaeger 1945, Allen 1952). The low incidence of January to February breeding by subadults may account

Table 11. Mean squares in age by month analysis[a] for ovarian and uterine weight of gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967.

Source of variation	Degrees of freedom	Log_{10} paired ovarian weight ($\underline{X\ 10^{-2}}$)	Log_{10} uterine weight ($\underline{X\ 10^{-1}}$)
Age	1	29.5**	4.2**
Month	10	5.4*	8.7**
Age x month	10	8.4**	3.6*
Error	83	2.2	1.7

[a] July was deleted from the analysis because there were no subadults collected during that month.

*$P<0.05$

**$P<0.01$

Table 12. Monthly mean paired ovarian and uterine weights (± standard error) of subadult and adult gray squirrels in Blacksburg, Virginia from December 1966 - November 1967.

Month	Subadults			Adults		
	No. of animals	Paired ovarian weight (mg)	Uterine weight (mg)	No. of animals	Paired ovarian weight (mg)	Uterine weight (mg)
Dec.	3	20.7 ± 3.1	148.2 ± 81.2	5	23.4 ± 2.8	210.1 ± 61.0
Jan.	3	14.9 ± 2.4	57.7 ± 30.0	3	25.9 ± 5.6	901.5 ± 390.0
Feb.	4	15.8 ± 6.4	78.6 ± 11.5	5	63.4 ±21.3	3028.5 ±1161.4
Mar.	5	17.5 ± 1.7	506.4 ± 259.6	5	30.0 ± 6.9	1865.8 ±1137.8
April	4	25.4 ± 6.1	226.8 ± 72.9	6	22.8 ± 4.9	331.0 ± 50.4
May	3	30.8 ± 2.8	188.9 ± 67.1	7	24.7 ± 1.7	483.3 ± 164.9
June	10	31.5 ± 2.6	747.5 ± 231.4	3	27.0 ± 3.9	1089.9 ± 732.7
July	0	--	--	4	49.0 ± 2.9	2798.3 ± 799.3
Aug.	4	32.1 ± 2.8	1713.1 ± 484.1	7	39.1 ± 3.2	4405.5 ±1641.9
Sept.	2	21.9 ± 3.9	221.5 ± 10.1	9	38.6 ± 2.5	1189.0 ± 434.2
Oct.	1	12.6	232.2	12	31.1 ± 2.9	662.0 ± 326.8
Nov.	3	27.3 ± 7.2	94.3 ± 46.0	1	22.4	239.8

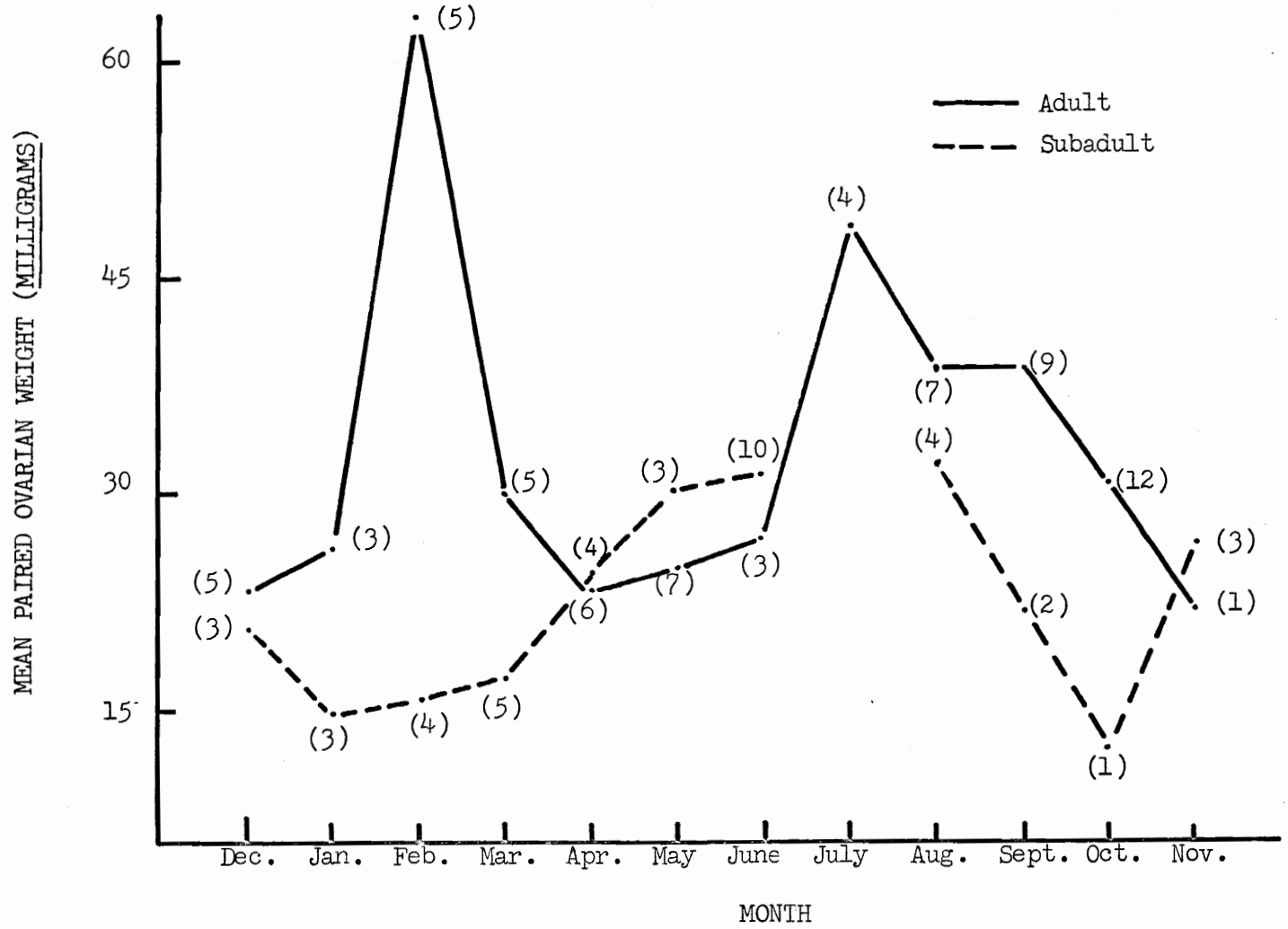

Fig. 1. Monthly mean paired ovarian weight for adult and subadult gray squirrels in Blacksburg, Virginia from December, 1966 to November, 1967. Number of animals is in parentheses.

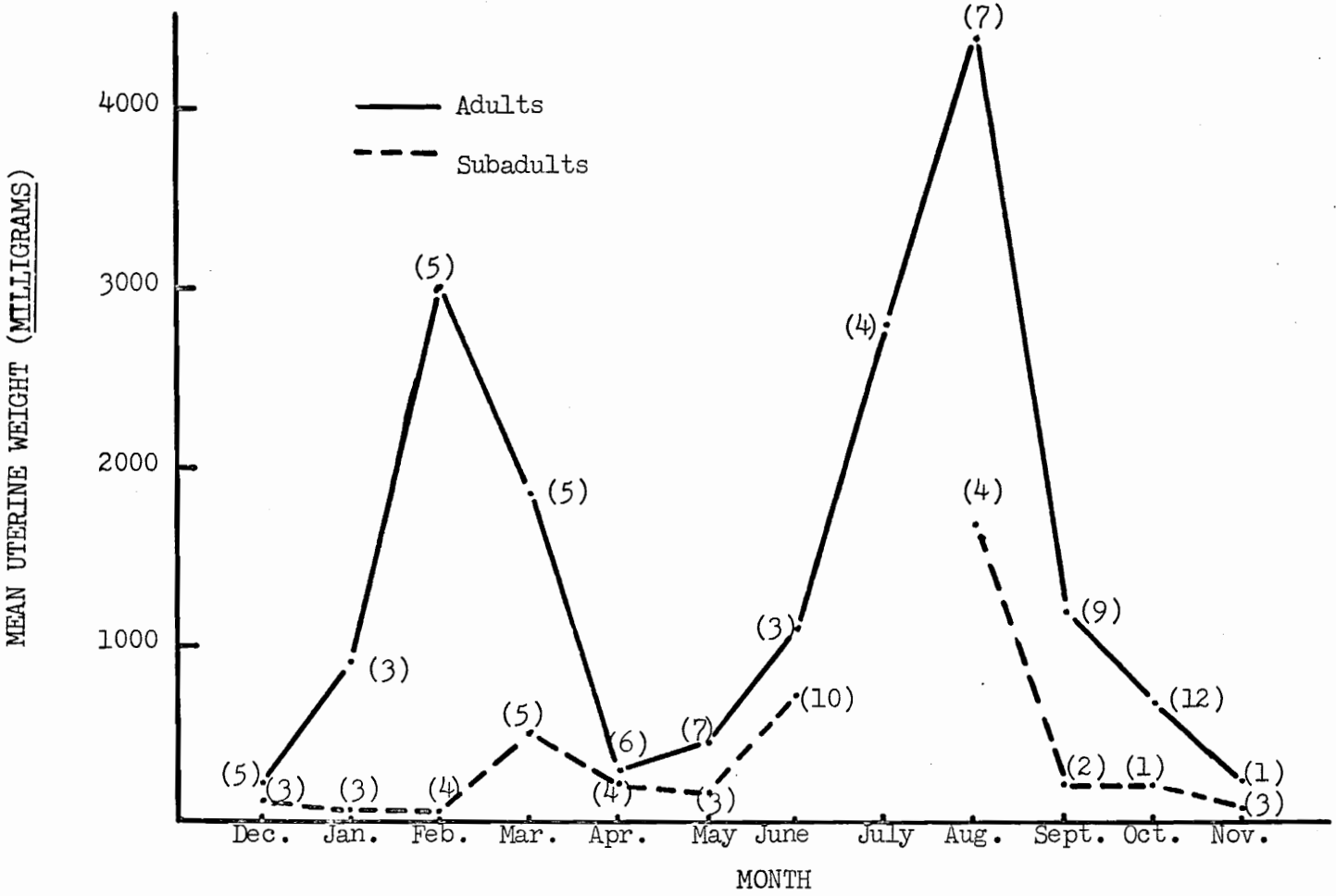

Fig. 2. Monthly mean uterine weight for adult and subadult gray squirrels in Blacksburg, Virginia from December, 1966 to November, 1967. Number of animals is in parentheses.

for the significant age by month interaction in ovarian weight and uterine weight. Differences between ages for these organ weights were most likely due to the fact that development in subadults was not as complete as in adults. Kirkpatrick (1974) reported similar age differences in deer.

Seasonal and age differences in number of tertiary follicles, sum of follicular diameters, or diameter of the largest follicle were not significant but were in the average diameter of the four largest follicles (Table 13). Although the results of the age by season analysis of these follicular data show few significant effects, it should be noted that lack of certain observations for July, November, and December required the deletion of these months from this analysis. Appendix Tables I and II show that seasonal differences were more evident for follicular measures of adults in the one-way analysis across months.

The number of tertiary follicles (Fig. 3) and sum of follicular diameters (Tables 14 and 15) followed the same seasonal pattern. In adults (Table 14) both were highest in July and lowest in April paralleling changes in ovarian weight to some extent. Unfortunately no subadults were collected in July, but the number of tertiary follicles and the sum of follicular diameters appeared to increase in spring and summer (Table 15). The decline in the number of tertiary follicles in January adults was probably due to the fact that both adults collected that month had open vaginas and could be considered near estrus. One of the three adults collected in June was in a similar

Table 13. Mean squares in age by month analysis[a] for follicular measurements of gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967.

Source of variation	Degrees of freedom	No. of tertiary follicles	Sum of follicular diameters ($\times 10^6$)	Diameter largest follicle ($\times 10^4$)	Log_{10} average diameter four largest follicles[b] ($\times 10^{-3}$)
Age	1	15.3	1.2	2.2	17.6*
Month	8	121.0	43.3	12.1	14.0**
Age x month	8	168.8	43.0	3.3	2.8
Error	66	120.7	31.9	6.1	3.8

[a] July and December were deleted from the analysis because subadult observations are lacking for those months. November deleted from the analysis because adult observations are lacking for that month.

[b] Error mean square has 56 degrees of freedom because squirrels with no tertiary follicles were deleted.

*$P<0.05$

**$P<0.01$

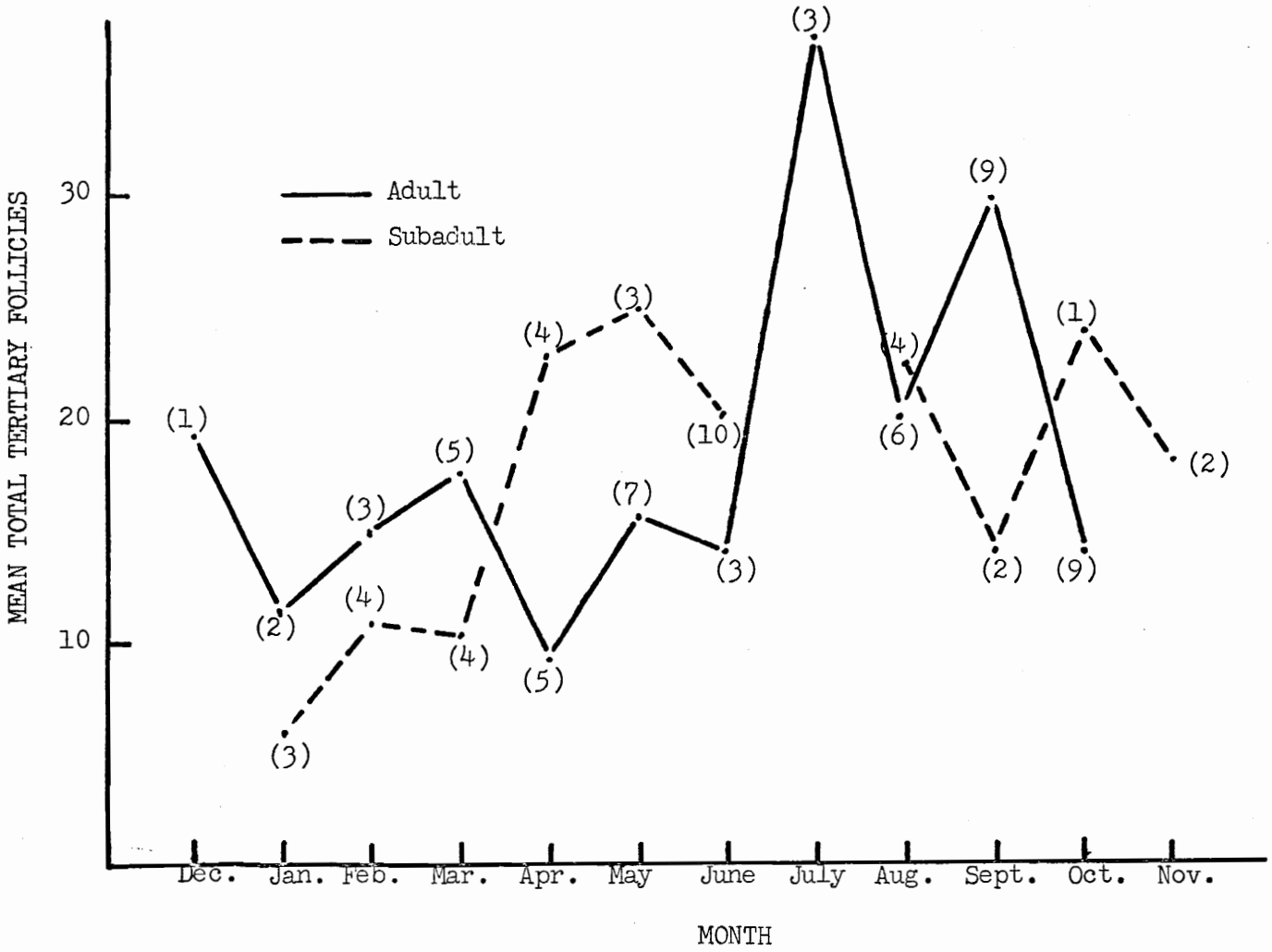

Fig. 3. Monthly mean total tertiary follicles for adult and subadult gray squirrels in Blacksburg, Virginia from December, 1966 to November, 1967. Number of animals is in parentheses.

Table 14. Monthly mean total tertiary follicles, diameter of the largest follicle and average diameter of the four largest follicles of adult gray squirrels in Blacksburg, Virginia from December 1966 - November 1967 (means ± standard error).

Month	No. of females	No. of tertiary follicles	Sum of follicular diameters (μm)	Diameter largest follicle (μm)	Average diameter four largest follicles (μm)
Dec.	1	19.0	10683.5	700.0	657.5
Jan.	2	11.5 ± 11.5	6350.8 ± 6350.7	460.0 ± 460.0	427.8 ± 427.7
Feb.	3	15.0 ± 7.6	8377.5 ± 4271.6	480.0 ± 241.7	465.3 ± 235.1
Mar.	5	17.6 ± 6.0	9628.0 ± 3345.3	702.0 ± 68.4	595.4 ± 53.8
April	5	9.0 ± 5.6	4853.7 ± 3121.9	520.0 ± 134.9	452.1 ± 118.6
May	7	15.9 ± 3.4	8748.2 ± 1738.0	615.7 ± 107.1	585.4 ± 101.6
June	3	14.3 ± 6.7	8233.2 ± 3338.2	906.7 ± 166.7	759.5 ± 48.0
July	3	37.0 ± 2.1	19212.5 ± 1037.0	773.3 ± 20.3	708.5 ± 7.9
Aug.	6	20.3 ± 4.7	11428.2 ± 2321.1	826.7 ± 90.3	735.5 ± 79.7
Sept.	9	29.7 ± 4.8	15924.8 ± 2153.4	756.7 ± 32.9	720.5 ± 27.3
Oct.	9	14.4 ± 3.4	7507.2 ± 1787.3	508.9 ± 99.1	474.4 ± 91.6
Nov.	0	--	--	--	--

Table 15. Monthly mean total tertiary follicles, diameter of the largest follicle and average diameter of the four largest follicles of subadult gray squirrels in Blacksburg, Virginia from December 1966 - November 1967 (means ± standard error).

Month	No. of females	No. of tertiary follicles	Sum of follicular diameters (μm)	Diameter largest follicle (μm)	Average diameter four largest follicles (μm)
Dec.	0	--	--	--	--
Jan.	3	6.0 ± 3.2	3207.0 ± 1769.6	430.0 ± 217.3	396.3 ± 200.6
Feb.	4	11.3 ± 5.4	6148.1 ± 2797.9	517.5 ± 175.0	481.9 ± 161.6
March	4	10.5 ± 3.8	4775.3 ± 1721.5	445.0 ± 149.0	403.1 ± 135.6
April	4	23.3 ± 6.2	11140.1 ± 2766.3	592.5 ± 14.4	572.0 ± 16.6
May	3	25.5 ± 2.8	13560.7 ± 1547.8	643.3 ± 13.3	627.5 ± 7.9
June	10	20.2 ± 3.1	11234.9 ± 1826.9	671.0 ± 35.0	593.5 ± 69.9
July	0	--	--	--	--
Aug.	4	22.3 ± 2.0	12975.6 ± 813.1	782.5 ± 51.5	747.5 ± 56.2
Sept.	2	14.0 ± 6.0	8337.0 ± 2505.1	720.0 ± 130.0	698.0 ± 103.5
Oct.	1	24.0	12144.0	630.0	585.5
Nov.	2	18.0 ± 1.0	9513.0 ± 342.5	610.0 ± 20.0	427.7 ± 427.7

state. Thus, as discussed previously, when squirrels in the sample near estrus, a reduction in numbers of follicles would be expected. Fig. 3 also shows declines in number of tertiary follicles during April and October, months when no pregnant animals were in the samples. On the other hand, the July peak for both variables was most likely related to the fact that all were pregnant. Squirrels of each reproductive state were present in the August Sample and this would tend to lower the means for number of follicles and sum of follicular diameters for that month.

The increase in the number of tertiary follicles of adult gray squirrels (Fig. 3) during February, March, and September is similar to the findings of Brauer and Dusing (1961). In the present study there was no month where considerable development of tertiary follicles could not be found in adults or subadults. However, one anestrous adult in January and two lactating adults, one each in April and October, had no tertiary follicles at all. Brauer and Dusing (1961) also found adults with no tertiary follicles.

Monthly means of the diameter of the largest follicle and of the average diameter of the four largest follicles generally followed similar seasonal patterns in both adults (Table 14) and subadults (Table 15). Fig. 4 shows that the average diameter of the four largest follicles tended to increase in the summer months for both age classes. In adults, this mean was highest in June when three animals were near estrus. A sharp decrease occurred from September to October when most of the animals examined were lactating. Thus, as described

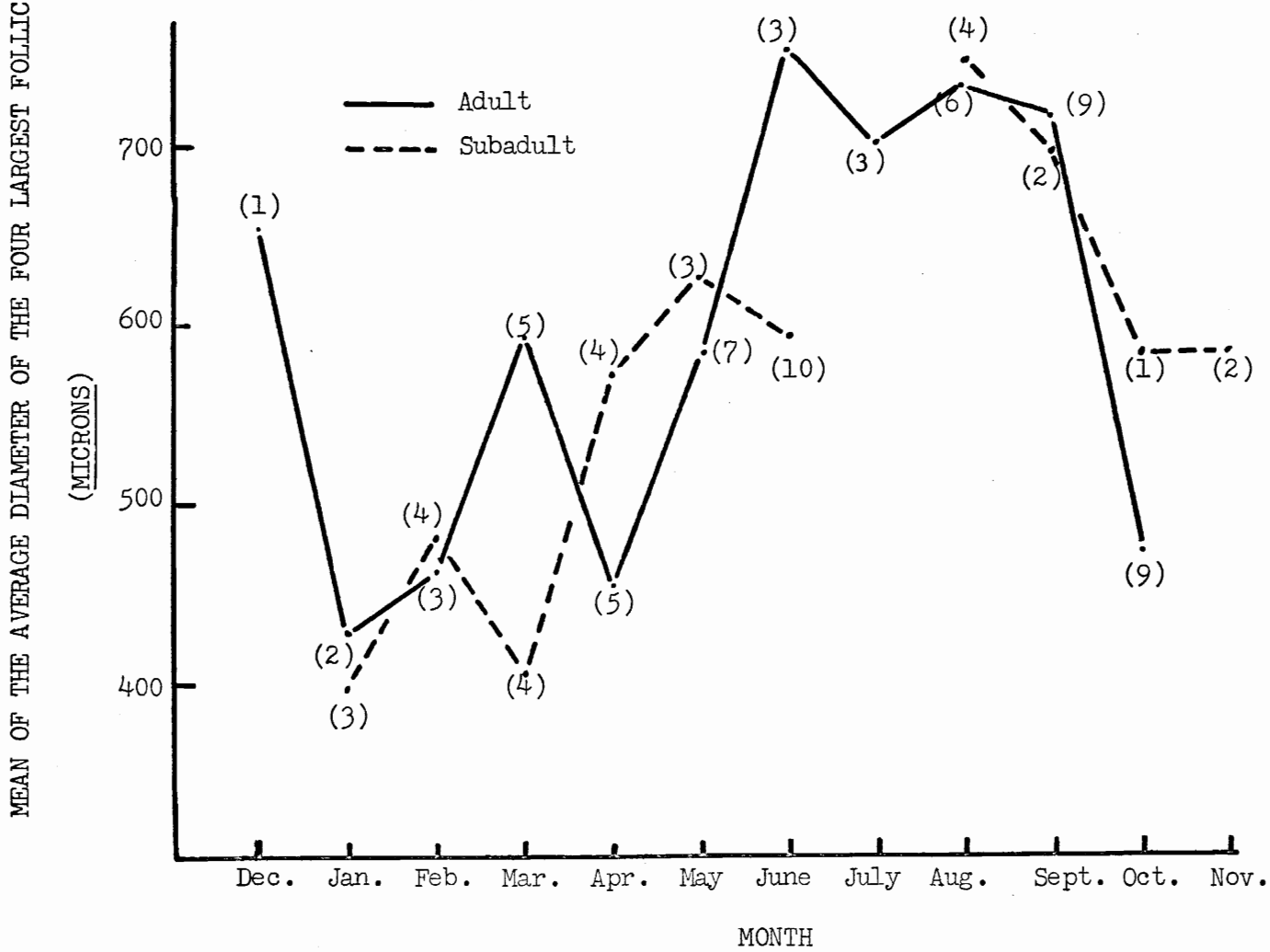

Fig. 4. Monthly mean of the average diameter of the four largest follicles of gray squirrels in Blacksburg, Virginia from December, 1966 to November, 1967. Number of animals is in parentheses.

for number of tertiary follicles and the sum of follicular diameters, seasonal changes in average diameter of the four largest follicles were also governed by the occurrence of the various reproductive states in the samples.

Luteal Counts and Prenatal Mortality

Comparisons between microscopic and macroscopic counts of luteal glands were made in order to determine the accuracy of the latter. Table 16 shows that macroscopic examination of pairs of ovaries following their collection in 1966 to 1967 revealed only 62 percent of the luteal glands actually present. These results indicate that macroscopic examination of ovaries, a method frequently used in making productivity estimates for many game species, may underestimate the actual frequency of ovulation. If it is impossible to obtain microscopic counts, macroscopic counts should be adjusted. As indicated here (Table 16), this adjustment could be at least 40 percent for the gray squirrel.

Subadults, adults, and the two ages combined had 28.6, 57.6 and 52.1 fetuses per 100 luteal glands respectively. These values are low because data from squirrels near estrus and in lactation were included. Consequently, luteal gland counts were high compared to fetal counts. For visibly pregnant squirrels only, there were 80.0, 82.9 and 82.6 fetuses per 100 luteal glands for subadults, adults, and the two ages combined, respectively. Considering all pregnant females, there was an average of 3.0 fetuses per female, with summer pregnancies (2.8 fetuses/female) averaging slightly lower than winter pregnancies (3.1 fetuses/

Table 16. Measures of productivity[a] of gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967. Number of females is in parentheses.

Age class	Number of luteal glands (microscopic exam)	Number of luteal glands (macroscopic exam)	Percent of luteal glands visible by macroscopic exam	Number of fetuses	Number of fetuses/100 luteal glands	No. of fetuses/100 luteal glands (pregnant females)
Subadult	14 (5)	11 (5)	78.6	4 (2)	28.6	80.0
Adult	59 (19)	35 (19)	59.4	34 (10)	57.6	82.9
Combined ages	73 (24)	46 (24)	62.0	38 (12)	52.1	82.6

[a]Luteal and fetal counts only from females with both ovaries available.

female) (Table 7).

Data presented by Smith (1967) for pregnant subadult and adult gray squirrels in North Carolina, revealed 84.5 fetuses per 100 luteal glands, approximately the same as found in this study. Although Smith (1967) was not able to identify luteal glands in lactating squirrels, both the results of this research and that of Deanesly and Parkes (1933) show that although regressed, they are easily identified in stained sections. The number of fetuses per female computed in this study are similar to those reported by Smith (1967) and Longley (1963) who found 3.6 and 3.5 fetuses per female, respectively, in combined winter and summer samples.

Reduction of productivity through prenatal loss was at least 17 percent prior to implantation. The average fetal and placental scar counts for both breeding periods combined were 3.2 fetuses per female and 2.6 placental scars per female. This indicates an approximate 18 percent post-implantation loss of young (Table 10). Similar prenatal loss was observed by Smith (1967).

Captive Squirrels

Experiment 1

Significant differences among treatments were found in uterine weight, average diameter of the largest follicle, average diameter of the four largest follicles and number of luteal glands (Tables 17 and 18). Duncan's New Multiple Range Test showed that mean uterine weights for Groups 2 and 4 were significantly higher ($P<0.05$) than those for Group 1 (Table 19). The number of luteal glands was signi-

Table 17. Mean squares for ovarian and uterine response of captive gray squirrels to exogenous hormone treatment in Experiment 1.

Source of variation	Degrees of freedom	Paired ovarian weight	Uterine weight ($\underline{X\ 10^5}$)	Ovarian index	Uterine index
Treatment	3	339.5	6.9*	9.8	3.9
Error	8	190.5	1.8	3.5	1.8

*$P<0.05$

Table 18. Mean squares for follicular response of captive gray squirrels to exogenous hormone treatment in Experiment 1.

Source of variation	Degrees of freedom	No. of tertiary follicles	Sum of follicular diameters ($\times 10^7$)	Diameter largest follicle ($\times 10^4$)	Average diameter four largest follicles ($\times 10^4$)	No. of luteal glands
Treatment	3	91.6	2.5	21.0*	15.0*	197.0**
Error	8	119.5	3.2	4.6	3.7	29.2

*$P<0.05$

**$P<0.01$

ficantly greater ($P<0.05$) for Groups 3 and 4 than for Group 1 (Table 20).

Means for all variables were lowest in the saline-treated controls (Tables 19 and 20). Group 2, which received FSH only, had the greatest number of tertiary follicles. Even though these animals received no HCG, they averaged 9.7 luteal glands per animal. This value was lower than the means for Groups 3 and 4 (19.3 and 13.3 respectively). The diameter of the largest follicle, average diameter of the four largest follicles and number of luteal glands were highest for Group 3.

It is apparent from these results that FSH-P alone as used in this experiment was sufficient to cause the formation of luteal glands. It is well known that the extraction method of Steelman et al. (1953) produces an FSH fraction slightly contaminated with LH. Literature supplied by the Armour-Baldwin Laboratories with their FSH-P indicates this method of extraction was utilized, but they supply no assay of LH contamination. Therefore, it was likely that much of the formation of the luteal glands in Group 2 of this experiment was a result of an unknown amount of LH in this FSH preparation.

As reviewed previously, luteinization of tertiary follicles accompanied by retention of ova occurs frequently in hormone-treated animals (Hammond 1952, Ingram 1962, Lostroh and Johnson 1966) and occasionally in wild squirrels (Deanesly and Parkes 1933). In this experiment (and Experiment 2 as well) all groups that demonstrated the formation of luteal glands had the retention of ova in many (Appendix Fig. I and Appendix Fig. II). Luteal structures should not be interpreted as evidence of an actual ovulation in these experiments. In

Table 19. Ovarian and uterine response of captive gray squirrels to exogenous hormone treatment in Experiment 1 (means ± standard error).

Group	Treatment[e]	females	Paired ovarian weight (mg)	Uterine weight (mg)	Ovarian index (mm)	Uterine index (mm)
1	Control	3	32.5 ± 4.2[a]	373.5 ± 157.1[b]	18.2 ± 0.3[a]	4.4 ± 0.9[a]
2	FSH	3	48.7 ± 7.7[a]	1379.9 ± 406.6[a]	20.6 ± 1.1[a]	6.5 ± 1.2[a]
3	FSH+HCG[c]	3	58.1 ± 12.6[a]	1014.3 ± 101.6[ab]	22.2 ± 1.6[a]	6.6 ± 0.3[a]
4	FSH+HCG[d]	3	49.2 ± 4.4[a]	1406.2 ± 186.7[a]	21.8 ± 0.9[a]	6.9 ± 0.5[a]

[a,b] Means in a column bearing the same superscript are not significantly different ($P<0.05$).
[c] HCG injected subcutaneously
[d] HCG injected intravenously
[e] See Table 2 for detailed description of treatment regimes.

Table 20. Follicular response of captive gray squirrels to exogenous hormone treatment in Experiment 1 (means ± standard error).

Group	Treatment[e]	Number of females	No. of tertiary follicles	Sum of follicular diameter (μm)	Diameter largest follicle (μm)	Average diameter four largest follicles (μm)	No. of luteal glands
1	Control	3	9.3± 5.3[a]	5428.7±3092.3[a]	666.7±60.6[a]	609.5± 52.5[a]	0.0[a]
2	FSH	3	22.0±10.1[a]	12479.0±4722.1[a]	833.3±173.8[a]	792.5±153.5[a]	9.7±3.7[ab]
3	FSH+HCG[c]	3	11.7± 0.9[a]	9743.8±1396.3[a]	1276.7± 74.6[b]	1137.5± 99.2[a]	19.3±4.7[b]
4	FSH+HCG[d]	3	13.3± 5.4[a]	9606.7±3011.8[a]	1060.0±146.4[ab]	954.5±113.9[a]	13.3±1.9[b]

[a,b]Means in a column bearing the same superscript are not significantly different ($P<0.05$).
[c]HCG injected subcutaneously.
[d]HCG injected intravenously.
[e]See Table 2 for detailed description of treatment regimes.

fact, luteinization of follicles without ovulation was more likely in most cases. Many of the luteal glands formed during these experiments (Appendix Fig. III) and in those of Mellace (1973) were located in the ovarian medulla and failed to have an apparent site of ovum release. The site of release is frequently indicated by the protrusion of luteal cells from the ovarian surface in wild squirrels (Appendix Fig. IV).

The greater number of luteal glands for Groups 2, 3 and 4 compared to the approximately 3 per female observed in wild squirrels (Table 16) indicates an excessive ovarian response occurred. Mean number of tertiary follicles in wild adult gray squirrels near estrus was 8.7 per female (Table 8). In all the hormone treated groups the number of tertiary follicles (Table 20) was in excess of this figure. This further indicated an abnormal response.

Due to probable LH activity in the FSH preparation, it is unclear as to what extent HCG was acting as a factor in luteal formation. Comparison of results in luteal development among Groups 2, 3, and 4 indicate that it may have caused additional luteinization in Groups 3 and 4.

There were no significant differences in any variable between the two methods of HCG injection. Although larger samples would yield more conclusive results, it is probable that subcutaneous injection of HCG produces a response as great as intravenous injection. Location of the vein for intravenous injection required more time per squirrel than needed for subcutaneous injection even though anesthetic was not

necessary for either. Therefore, it is recommended that subcutaneous injection of HCG be utilized in future research.

Experiment 2

Few significant differences were found between treatments in Experiment 2. Uterine index and number of luteal glands showed significant differences due to treatment at $P<0.05$ and $P<0.01$ respectively (Tables 21 and 22). Duncan's New Multiple Range Test showed the uterine index for Group 3 to be significantly higher ($P<0.05$) than that of Group 1 (Table 23). The number of luteal glands was significantly higher in Group 3 than in Groups 1 and 2 (Table 24).

These results indicate that NIH-FSH produces considerable follicular growth without luteinization of follicles such as occurred in all applications of FSH-P in this experiment and in Experiment 1. Squirrels in Group 1 had, in addition to other tertiary follicles, 6 extremely large tertiary follicles in size classes 30 to 32, 4 of which were in excess of 1400 μm average diameter. It appears that NIH-FSH lacked sufficient LH content to cause luteinization in the same time as did FSH-P, but continued to provide a stimulus to growing follicles allowing achievement of a large size prior to sacrifice. Richardson (1967) and Perry (1971) state that normal growth of follicles is accompanied by physiological changes of the theca interna which make it sensitive to an ovulatory stimulus. Since the large follicles of animals in Group 1 failed to respond to the HCG injection, it is likely that such physiological changes did not occur.

The application of 2 mg of FSH-P per day did not result in major

Table 21. Mean squares for ovarian and uterine response of captive gray squirrels to exogenous hormone treatment in Experiment 2.

Source of variation	Degrees of freedom	Paired ovarian weight	Uterine weight ($\underline{X\ 10^{5}}$)	Ovarian index	Uterine index
Treatment	2	58.7	5.6	0.5	4.9*
Error	4	255.8	2.8	6.4	0.4

*$P < 0.05$

Table 22. Mean squares for follicular response of captive gray squirrels to exogenous hormone treatment in Experiment 2.

Source of variation	Degrees of freedom	No. of tertiary follicles	Sum of follicular diameters ($\times 10^7$)	Diameter largest follicle ($\times 10^4$)	Average diameter four largest follicles ($\times 10^4$)	No. of luteal glands
Treatment	2	51.2	5.8	6.2	8.5	62.9**
Error	4	74.1	3.4	2.2	2.8	2.5

**P<0.01

Table 23. Ovarian and uterine response of captive gray squirrels to exogenous hormone treatment in Experiment 2 (means ± standard error).

Group	Treatment[c]	Number of females	Paired ovarian weight (mg)	Uterine weight (mg)	Ovarian index (mm)	Uterine index (mm)
1	200 µg NIH-FSH+HCG	3	60.7± 5.8[a]	204.1± 68.9[a]	21.3±0.8[a]	3.6±0.3[a]
2	200 µg FSH-P+HCG	2	58.2±14.0[a]	863.3±707.3[a]	21.8±2.5[a]	5.2±0.8[ab]
3	2 mg FSH-P+HCG	2	50.9±14.7[a]	1116.8±197.8[a]	20.8±2.1[a]	6.5±0.1[b]

[a,b] Means in a column bearing the same superscript are not significantly different ($P<0.05$).

[c] See Table 3 for detailed description of treatment regimes.

Table 24. Follicular response of captive gray squirrels to exogenous hormone treatment in Experiment 2 (means ± standard error).

Group	Treatment[c]	Number of females	No. of tertiary follicles	Sum of follicular diameters (μm)	Diameter largest follicle (μm)	Average diameter four largest follicles (μm)	No. of luteal glands
1	200 μg NIH-FSH+HCG	3	25.0± 4.0[a]	20310.5±2380.8[a]	1326.7± 78.8[a]	1266.5± 96.7[a]	0.0[b]
2	200 μg FSH-P+HCG	2	19.5± 0.5[a]	13104.7± 802.3[a]	1150.0± 20.0[a]	954.5± 9.0[a]	2.0±2.0[b]
3	2 mg FSH-P+HCG	2	16.0±10.0[a]	11330.0±7043.0[a]	1010.0±160.0[a]	950.0±166.5[a]	10.0±1.0[a]

[a,b]Means in a column bearing the same superscript are not significantly different ($P<0.05$).

[c]See Table 3 for detailed description of treatment regimes.

differences in luteal formation when compared to squirrels treated with 4 mg on alternate days in Mellace's (1973) fifth experiment. It has been demonstrated in Group 2 of the present experiment that FSH-P in dosage levels one-tenth of those utilized in Group 3 (and Groups 2, 3, and 4 of Experiment 1) also can produce as great an ovarian response if applied on a daily basis. Therefore increased handling and subsequent stress on the squirrels due to daily injection does not appear to reduce ovarian response of treated squirrels.

Another possible factor related to the extent of luteinization is the day of sacrifice in relation to the last day of FSH administration. It has been shown in Experiment 1 that FSH-P alone will cause luteinization. The increased luteinization of animals sacrificed or laparotomized on the fifteenth day (first day of FSH = Day 1) may have been due to a longer interval for FSH-P to manifest itself.

In Mellace's (1973) experiments using FSH-P, the only squirrels that had luteal glands were those treated with HCG on the thirteenth day and laparotomized on the fifteenth day. The results in Group 1 of Experiment 2 of the present study showed that even with adequate follicular development, the HCG failed to cause luteinization. Since animals in Groups 2 and 3 had similar follicular development (Table 24), it is possible that the significant difference in average number of luteal glands was due to the longer interval after FSH treatment before sacrifice in Group 3.

The apparent remains of two ova were found in the oviduct of squirrel #2071 in Group 2 for which a total of 4 luteal glands had been

observed (Appendix Fig. V and Appendix Fig. VI). These remains averaged 24 μm and 17 μm in diameter. The mean of the average diameter of 10 oocytes measured in tertiary follicles of wild squirrels was 23 μm (range = 17-28 μm), relatively close to the above diameters. The structures found in the oviduct of this squirrel were similar in pigmentation and shape to atretic oocytes observed in wild squirrels (Appendix Fig. VII). They also were similar to entrapped ova of treated animals (Appendix Fig. II). Although not conclusive evidence, this suggests that ovulation probably occurred in some gray squirrels treated with exogenous hormones. Further proof awaits an improved method of ova collection for this species.

The ovaries of squirrel #4038 failed to show any tertiary, secondary, primary or primordial follicles (Appendix Fig. VIII). Senescent cow (Bos taurus, Erickson 1966) and mouse (Mus musculus, Jones and Krohn 1961) ovaries have very few or none of these structures. In the female mouse (M. musculus) this disappearance occurs at an age of 450 days (Jones and Krohn 1961). It is possible that this squirrel, which was captured on the Virginia Polytechnic Institute and State University campus, had outlived its reproductive usefulness. It is also possible that it never had any of these ovarian elements to begin with.

Ovarian and Uterine Indices

A review of the experiments in the present study suggests that use of the ovarian and uterine indices (Mellace 1973) has limited value. In Experiment 1 significant differences among the groups were demonstrated in the various follicular and luteal measures but not in the

ovarian index or the uterine index (Tables 17 and 18). In Experiment 2 the uterine index showed significant differences among groups (Table 21). However this tells little about the ovarian changes which are of primary concern. In Mellace's (1973) last experiment, although differences in luteal formation occurred, the uterine and ovarian indices were not reported as showing significant differences. Thus, there appears to be little correspondence between measures of ovarian and uterine size and the important morphological changes taking place in the ovary. Measurements of histological characteristics such as diameters and numbers of follicles (or luteal glands) provide a more refined index of response to exogenous gonadotropin.

SUMMARY AND CONCLUSIONS

Significant differences among reproductive states of wild female gray squirrels, Sciurus carolinensis, were found in paired ovarian weight, uterine weight, number of tertiary follicles, sum of follicular diameters and the average diameter of the four largest follicles. In general, pregnant squirrels or those with an open vaginal orifice had the highest means for paired ovarian weight, uterine weight and the average diameter of the four largest follicles. Animals with a closed vaginal orifice or lactating had lower means for those variables. Numbers of tertiary follicles were lowest in adults with an open vaginal orifice.

Significant seasonal differences existed in paired ovarian weight, uterine weight, and the average diameter of the four largest follicles. A significant effect due to age was observed for these variables. However, both ages generally followed the same seasonal trends. Ovarian and uterine weights increased in late winter, decreased in early spring, increased during the summer months and decreased again in the fall. Follicular changes were more variable, although gradual increases appeared during the summer months. These seasonal trends reflected the occurrence of the various reproductive states in the monthly samples.

Macroscopic counts of luteal glands in gray squirrel ovaries should be avoided due to potential inaccuracy. Only 62 percent of luteal glands actually present were observed macroscopically. Prenatal losses prior to implantation were at least 17 percent as indicated by

comparison of number of luteal glands per female to number of fetuses per female. An approximate 18 percent post-implantation loss of young was indicated by comparison of number of fetuses per female to number of placental scars per female.

In Experiment 1, FSH-P alone caused the luteinization of tertiary follicles in captive gray squirrels. There were no apparent differences in ovarian and uterine response of gray squirrels receiving HCG subcutaneously compared to those receiving HCG intravenously. It is recommended that subcutaneous application of HCG be utilized in future experiments.

In experiment 2, low dosages (200 µg) of FSH-P and NIH-FSH-S-6 in a regime patterned after the methods of Williams et al. (unpublished) produced as great a follicular response as high dosages (2 mg) of FSH-P in the regime utilized by Mellace (1973). No luteinization of follicles occurred in animals treated with NIH-FSH-S-6. Squirrels treated with the regime developed by Mellace (1973) had significantly ($P<0.01$) more luteal glands than those treated with the regime utilized by Williams et al. (unpublished).

Although retention of ova occurred in many of the luteal glands formed during these experiments, the remains of what were probably two ova were found in the oviduct of a treated squirrel. Thus, successful induction of ovulation may have occurred. LH contamination of FSH-P probably enhanced the effect of HCG application in these and previous experiments.

Results of these experiments suggest that the use of the ovarian and uterine size indices (Mellace 1973) are of limited value. Histolo-

gical methods of quantification of follicular and luteal changes are better indices of ovarian response to exogenous gonadotropins.

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APPENDIX

Appendix Table I. Mean squares of ovarian and uterine measurements in analysis of effect due to month for adult gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967. Degrees of freedom are in parentheses.

Source of variation	Log_{10} paired ovarian weight ($\times 10^{-2}$)	Log_{10} uterine weight ($\times 10^{-1}$)	No. of tertiary follicles ($\times 10^{2}$)	Sum of follicular diameter ($\times 10^{7}$)	Diameter largest follicle ($\times 10^{4}$)	Log_{10} average diameter four largest follicles[a] ($\times 10^{-3}$)
Month	9.3 (11)**	8.1 (11)**	3.0 (10)*	8.2 (10)*	9.8 (10)	8.9 (10)
Error	2.5 (55)	1.9 (55)	1.4 (42)	3.7 (42)	6.9 (42)	4.4 (36)

[a]Animals with no tertiary follicles were deleted from the analysis

*$P < 0.05$

**$P < 0.01$

Appendix Table II. Mean squares of ovarian and uterine measurements in analysis of effect due to month for subadult gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967. Degrees of freedom are in parentheses.

Source of variation	Log_{10} paired ovarian weight ($\underline{X\ 10^{-2}}$)	Log_{10} uterine weight ($\underline{X\ 10^{-1}}$)	No. of tertiary follicles ($\underline{X\ 10^{2}}$)	Sum of follicular diameter ($\underline{X\ 10^{7}}$)	Diameter largest follicle ($\underline{X\ 10^{4}}$)	Log_{10} average diameter four largest follicles[a] ($\underline{X\ 10^{-3}}$)
Month	7.0 (10)**	7.0 (10)**	1.4 (9)	4.5 (9)	4.7 (9)	5.8 (9)*
Error	1.5 (31)	1.2 (31)	7.8 (27)	2.1 (27)	4.1 (27)	2.4 (23)

[a]Animals with no tertiary follicles were deleted from the analysis.

*P<0.05

**P<0.01

Appendix Table III. Mean squares of ovarian and uterine measurements in analysis of effect due to reproductive status for adult gray squirrels collected in Blacksburg, Virginia from December 1966 - November 1967. Degrees of freedom are in parentheses.

Source of variation	Log_{10} paired ovarian weight ($\times 10^{-2}$)	Log_{10} uterine weight ($\times 10^{-1}$)	No. of tertiary follicles ($\times 10^{2}$)	Sum of follicular diameter ($\times 10^{7}$)	Diameter largest follicle ($\times 10^{4}$)	Log_{10} average diameter four largest follicles[a] ($\times 10^{-3}$)
Reproductive status	3.3 (3)**	44.0 (3)**	6.7 (3)**	18.0 (3)**	13.0 (3)	28.0 (3)**
Error	0.2 (62)	0.9 (62)	1.4 (48)	3.8 (48)	7.3 (48)	3.9 (42)

[a]Animals with no tertiary follicles were deleted from the analysis.

**P<0.01

Appendix Table IV. Mean squares of ovarian and uterine measurements in analysis of effect due to reproductive status for subadult gray squirrels collected in Blacksburg, Virginia from December 1966 to November 1967. Degrees of freedom are in parentheses.

Source of variation	Log_{10} paired ovarian weight ($\times 10^{-2}$)	Log_{10} uterine weight ($\times 10^{-1}$)	No. of tertiary follicles ($\times 10^{1}$)	Sum of follicular diameter ($\times 10^{7}$)	Diameter largest follicle ($\times 10^{4}$)	Log_{10} average diameter four largest follicles[a] ($\times 10^{-3}$)
Reproductive status	5.6 (3)	1.9 (3)**	6.1 (3)	2.1 (3)	2.9 (3)	3.3 (3)
Error	2.6 (38)	0.1 (38)	9.7 (33)	2.8 (33)	4.4 (33)	3.3 (29)

[a]Animals with no tertiary follicles were deleted from the analysis.
**P<0.01

Appendix Table V. An account of all gray squirrels captured during this research.

Squirrel number	Sex	Date squirrel captured	Date squirrel died	Weight on capture (grams)	Weight on death (grams)
1774-1775[a]	M	10/15/73	1/21/74	808	437
2334-2335[a]	M	10/15/73	1/22/74	540	512
2436-2437[a]	M	10/22/73	1/18/74	739	490
2476-2477[c]	F	10/25/73	7/23/74	470	478
2482-2483[b]	F	11/ 9/73	4/16/74	--	533
2484-2495[d]	M	11/21/73	12/ 3/73	580	320
2486-2487[d]	M	11/21/73	11/28/73	530	335
2488-2489[a]	M	11/21/73	1/19/74	585	420
2490-2491[a]	M	11/21/73	1/23/74	500	533
2492-2493[a]	M	11/21/73	1/23/74	560	550
2063-2064[b]	F	11/22/73	4/16/74	540	520
2494-2495[b]	F	11/22/73	4/16/74	570	564
2496[a]	M	11/23/73	1/22/74	595	475
2497[b]	F	11/23/73	4/16/74	660	550
2498[a]	M	11/23/73	1/26/74	580	425
1391-1393[d]	F	11/25/73	12/ 3/73	360	250
1396-5806[b]	F	11/25/73	4/16/74	530	571
5812-5824[b]	F	12/ 3/73	4/16/74	760	625
3376-3379[d]	F	1/10/73	1/20/74	438	300
3381[b]	F	1/13/74	4/16/74	620	638
2306[b]	F	1/16/74	4/16/74	560	602
3378[b]	F	1/16/74	4/16/74	380	354
3379[b]	F	1/19/74	4/16/74	--	557
3340-3341[d]	F	1/25/74	2/ 1/74	--	359
3332-3333[b]	F	1/27/74	4/16/74	--	556
4000-4001[b]	F	2/16/74	4/16/74	602	563

Appendix Table V. An account of all gray squirrels captured during this research (continued).

Squirrel number	Sex	Date squirrel captured	Date squirrel died	Weight on capture (grams)	Weight on death (grams)
2071-2072[c]	F	3/28/74	7/23/74	620	605
3382-3383[d]	F	4/ 2/74	4/ 6/74	516	--
3384-3385[c]	F	4/ 3/74	7/30/74	593	537
4039[c]	F	4/11/74	7/30/74	579	558
4002[c]	F	5/ 1/74	7/23/74	437	556
4021[c]	F	5/ 1/74	7/23/74	553	512
4038[c]	F	5/17/74	7/23/74	532	--
4006-4007[c,e]	F	6/ 9/74	--	679	--
4015-4016[c]	F	6/10/74	7/23/74	490	474

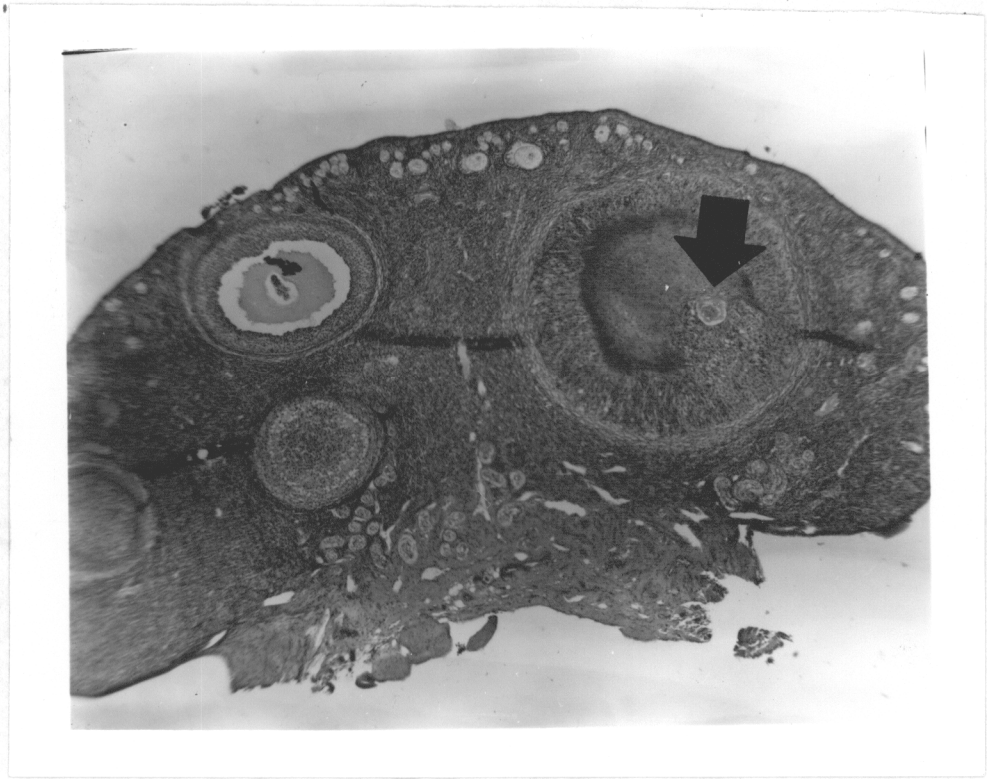
[a]Used in experiment described by Merson et al. (1974).

[b]Used in Experiment 1 of the present study.

[c]Used in Experiment 2 of the present study.

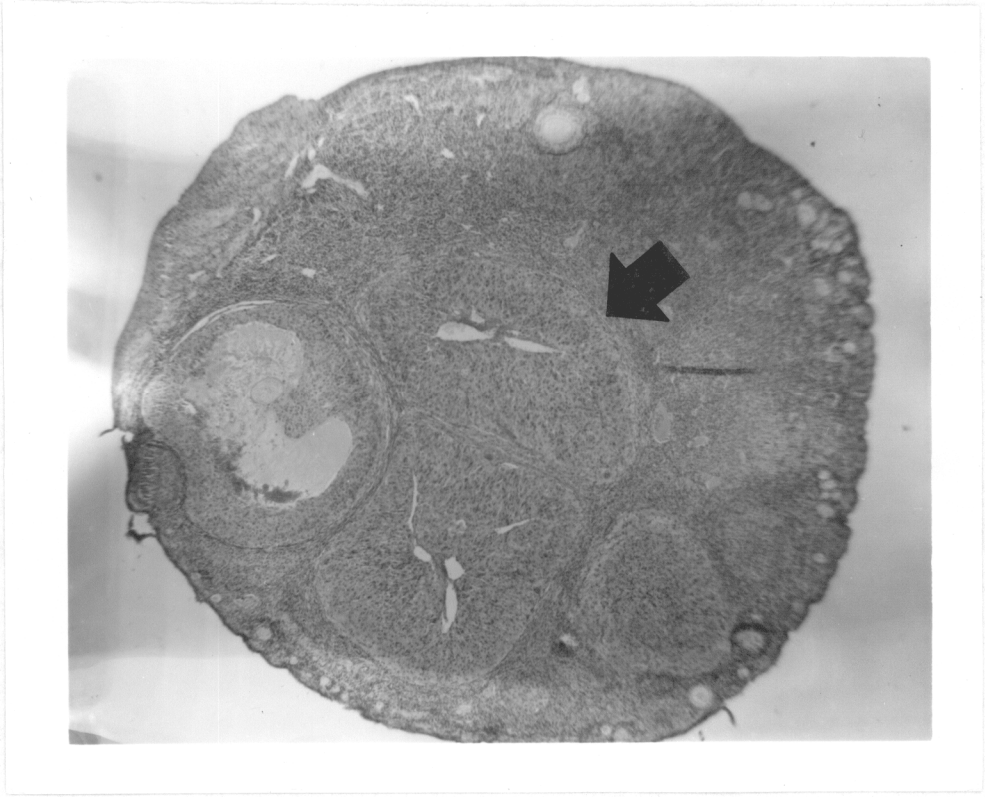
[d]Probable "shock" death.

[e]Two young were born to this female on 7/18/74. They were weaned on 9/12/74 and had the following body weights: #4049, a male - 195 g; #4050, a female - 201 g. Both young were alive at this writing (12/17/74).

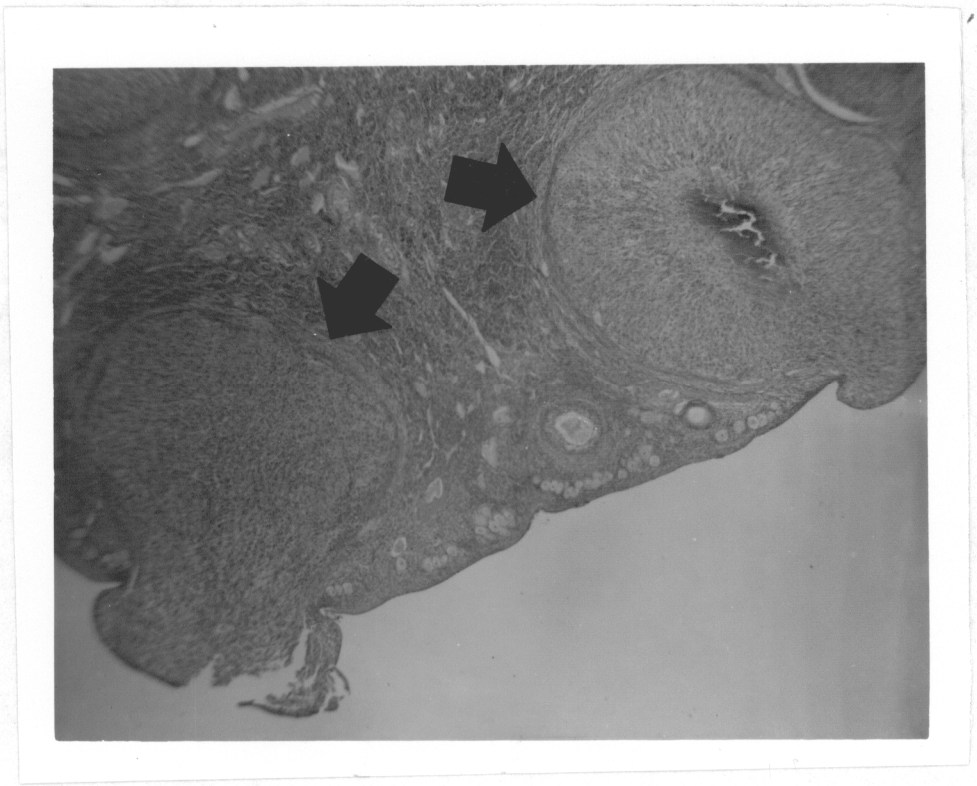


Appendix Fig. I. A retained ovum in a luteal gland of a captive gray squirrel (#2497, Experiment 1, Group 2) treated with FSH-P (X 40).

Appendix Fig. II. Close-up of a retained ovum in a luteal gland of a captive gray squirrel (#2497, Experiment 1, Group 2) treated with FSH-P (X 450).

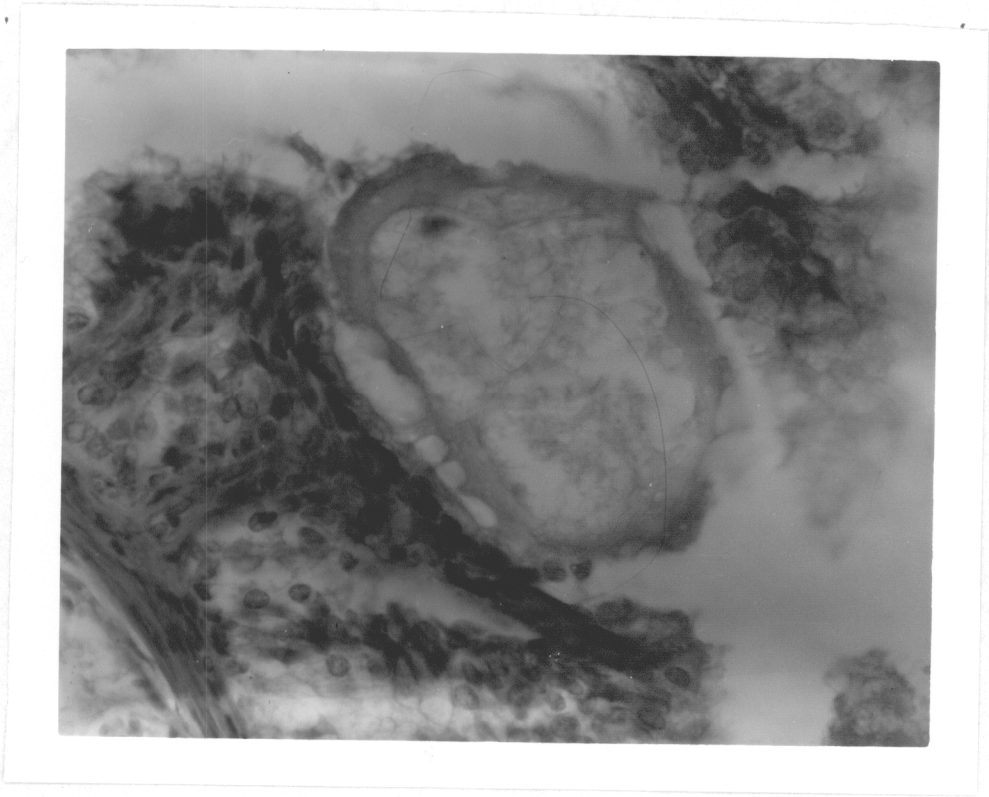


Appendix Fig. III. The ovary of a captive gray squirrel treated with FSH-P and intravenous HCG (#3381, Experiment 1, Group 4). A luteal gland is located in the ovarian medulla (X 40).

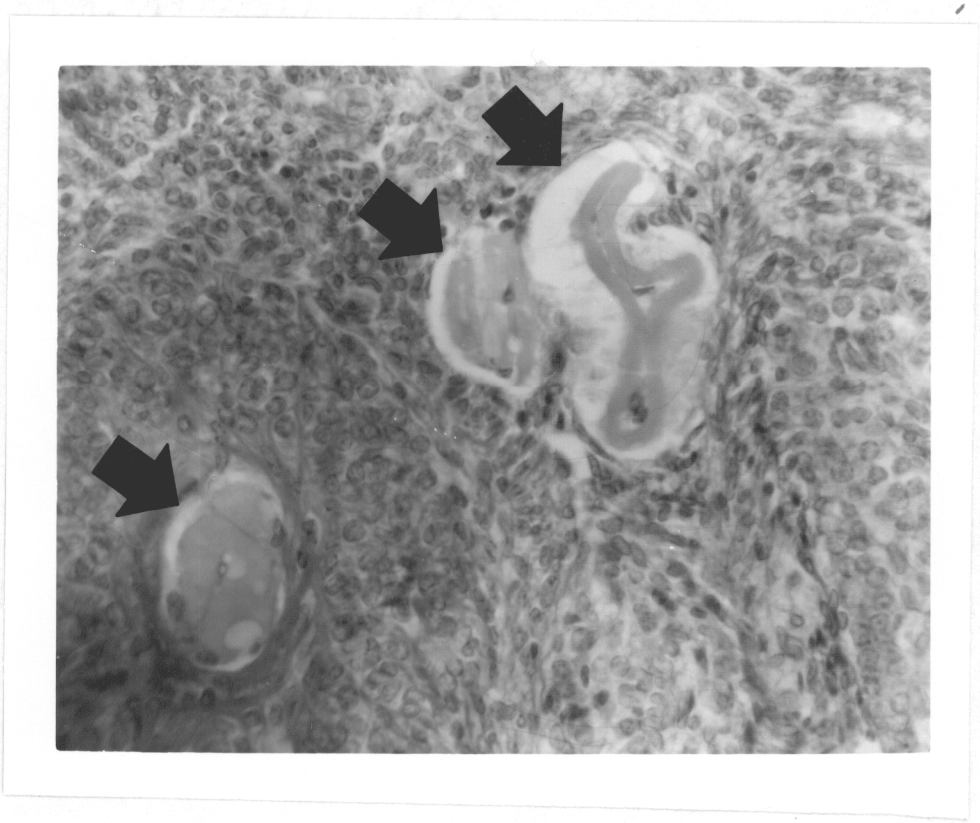


Appendix Fig. IV. Two recently formed luteal glands of a wild gray squirrel that had a perforate vaginal orifice when collected (#62, June 1967, X 40).

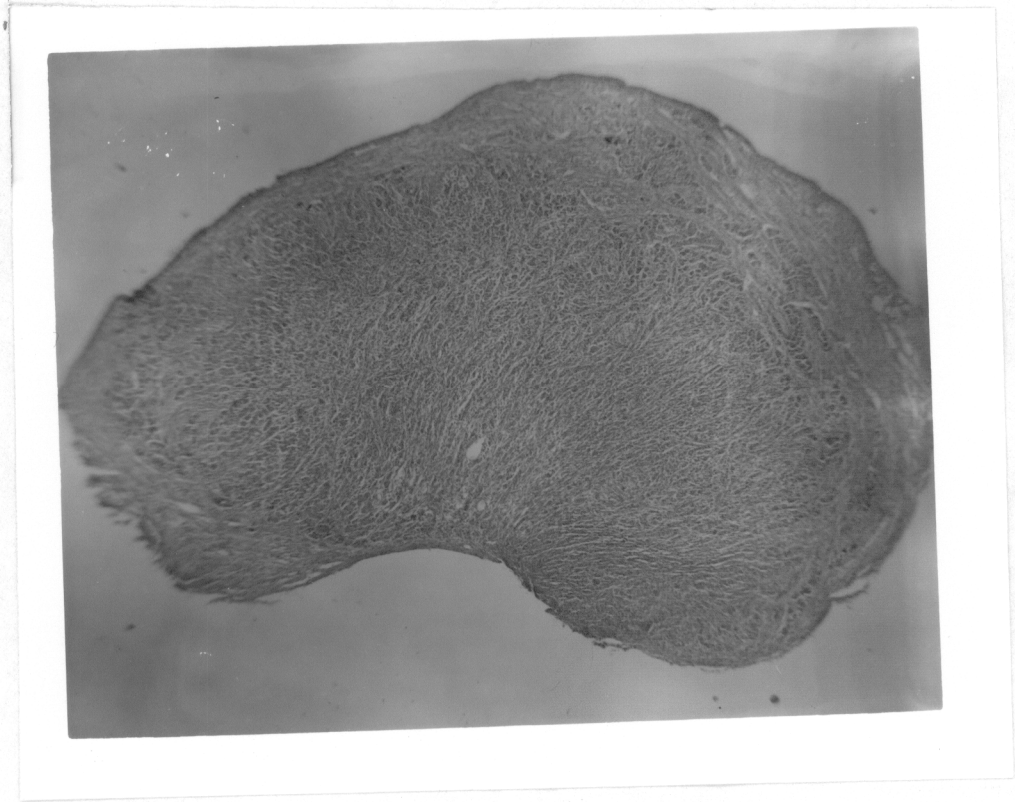
Appendix Fig. V. Cross-section of an oviduct of a captive gray squirrel treated with FSH-P and subcutaneous HCG (#2071, Experiment 2, Group 2). The probable remains of two ova are visible in the lumen (X 100).



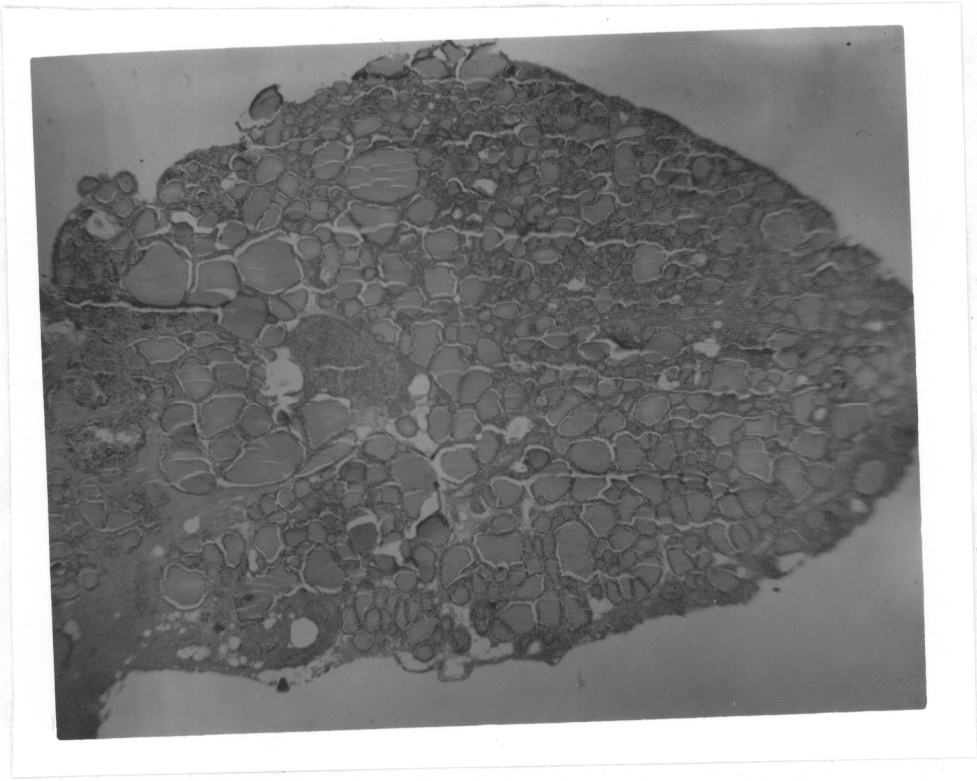
Appendix Fig. VI. Close-up of one of the structures found in the oviduct of a captive gray squirrel treated with FSH-P and subcutaneous HCG (#2071, Experiment 2, Group 2, X 450).



Appendix Fig. VII. Atretic oocytes in the stroma of an ovary removed from a wild gray squirrel (#65, June 1967, X 450).



Appendix Fig. VIII. One of the ovaries lacking oocytes and follicles found in a captive gray squirrel (#4038, X 40).



Appendix Fig. IX. The ovary of a wild gray squirrel showing abnormal secretory activity. The other ovary of this animal was normal (#48, April 1967, X 40).

VITA

Cleveland John Cowles, son of Viola H. and John T. Cowles, was born in San Antonio, Texas on July 16, 1947. He attended public schools in Glenshaw, Pennsylvania and graduated from Shaler High School in 1965. The author entered the College of Agriculture at the University of Maine, Orono, Maine in the fall of 1965. While at the University of Maine, he was a member of the Student Chapter of the Wildlife Society (President 1968-69); the honorary societies of Alpha Zeta, Xi Sigma Pi, and Phi Kappa Phi; and an employee of the Resident Counselor System. He graduated from the University of Maine in June 1969 receiving a Bachelor of Science in Wildlife Management with highest distinction.

In June 1969, the author married the former Maureen P. Streiff of Glenshaw, Pennsylvania. He was employed as a teacher of secondary science in Carmel, Maine and as a seasonal employee of the Maine Game Division until August 1971. In September 1971, the author assumed duties as a teacher of secondary science and mathematics in Chambersburg, Pennsylvania. A son, Todd, was born in August, 1972.

The author became a candidate for the Master of Science degree in Wildlife Management at Virginia Polytechnic Institute and State University in September 1973. While at V.P.I.&S.U., he was employed as a Graduate Teaching Assistant and as a Graduate Research Assistant. He is a member of the Wildlife Society and the National Wildlife Federation.

Cleveland J. Cowles

OVARIAN AND UTERINE CHANGES OF GRAY SQUIRRELS AS AFFECTED BY SEASON, AGE, REPRODUCTIVE STATE AND EXOGENOUS HORMONES

by

Cleveland J. Cowles

(ABSTRACT)

Sixty-seven adult and 42 subadult female gray squirrels (Sciurus carolinensis) were collected over a period of one year between December, 1966 and November, 1967 in the vicinity of Blacksburg, Virginia. Each squirrel was classified as being in one of four reproductive states (vaginal orifice closed, vaginal orifice open, pregnant, or lactating). Differences in ovarian and uterine characteristics due to month, age, and reproductive state were analysed by analysis of variance and a multiple range test.

Pregnant adults had significantly ($P<0.05$) greater paired ovarian weights than lactating adults or those adults with a closed vaginal orifice. Uterine weights in the four reproductive states were all significantly ($P<0.05$) different, with pregnant adults highest, adults with open vaginal orifices next highest, lactating adults next highest and adults with closed vaginal orifices lowest. Pregnant adults had significantly ($P<0.05$) more tertiary follicles and significantly ($P<0.05$) higher sums of follicular diameters than all other reproduc-

tive states. Squirrels with open vaginal orifices had significantly ($P<0.05$) higher average diameters of the four largest follicles than all other reproductive states.

Ovarian and uterine weights of subadults were less than adults during most months. Ovarian and uterine weights increased in late winter, decreased in early spring, increased during the summer and decreased in the fall. Follicular development was more variable, but showed general increases in the summer months. The seasonal trends reflected the frequency of occurrence of the various reproductive states in the monthly samples.

Two experiments were conducted on captive female gray squirrels. In the first experiment it was determined that FSH-P can cause the formation of luteal glands in the absence of HCG injection. LH contamination of FSH-P probably caused the luteinization. There were no significant differences in ovarian response between squirrels receiving HCG subcutaneously and those receiving it intravenously after pretreatment with FSH-P. In the second experiment, 2.0 mg FSH-P/day on days 1, 2, 3, 4, 5, 6, and 7 with subcutaneous injection of 200 IU HCG on day 13 produced significantly ($P<0.05$) more luteal glands than 200 μg FSH-P on days 1, 2, 3, 4, 5, 6, and 7 with subcutaneous injection of 200 IU HCG on day 5. However, there were no significant differences in development of tertiary follicles. Squirrels treated with NIH-FSH-S-6 and HCG had development of large tertiary follicles with no luteinization.