FURTHER STUDIES OF REPRODUCTION OF THE GROUND SQUIRREL (*CITELLUS TRIDECEMLINEATUS*) AND THE EFFECT OF HETERO ANTERIOR PITUITARY IMPLANTS

by

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INTRODUCTION

For the past eight years there have been kept in the animal house at the Kansas State College of Agriculture and Applied Science hundreds of thirteen-lined ground squirrels (Citellus tridecemlineatus). During this time we have on record, from matings in the laboratory, only six litters and another pregnancy accounted for by the presence of corpora lutea. This fact and the other studies on the ground squirrel in this laboratory by Johnson and Wade (1931) and by Johnson and Foster (unpublished) have shown the need for a detailed study of the reproductive cycle in captivity and of the effect of various controllable conditions on this cycle, in order to aid in understanding the lack of reproduction in the laboratory and the factors conducive to it in the field.

The purpose of this work, therefore, has been to study further the reproductive cycle in the laboratory, filling in gaps in the earlier work, to study the cycle in the female by the vaginal smear method, and to determine the effects of anterior pituitary implants, a limited food supply, a high protein diet, ultra-violet radiations, and hibernation, on the laboratory animals.

REVIEW OF LITERATURE

Drips (1919) states that the spermophiles of the typical variety (Citellus tridecemlineatus) go into hibernation about the middle of October. The males make their appearance as soon as they come out of hibernation, usually between April 10 and 25 in Minnesota. The breeding season comes shortly after the adult animals appear in the spring (Johnson, 1917; Drips, 1919; O. Wade, 1927). The rutting period lasts from two to four weeks, reaching its peak in April (Drips, 1919; O. Wade, 1927). At this time the testes are much "inflamed" and "swollen" (Wade, 1927). Ovulation occurs only after coitus, corpora lutea persisting until about September 1 (Drips, 1919). The females refused to breed a second time during the same season (Wade, 1927).

The males appear from hibernation before the females (Lee, 1903; O. Wade, 1927) for the thirteen-lined form. Shaw (1926) found the same thing for the Columbian ground squirrel. Johnson (1917) reports that the time of hibernation of Citellus tridecemlineatus may vary with the weather conditions.

The gestation period for the typical variety was found to be 28 days (Drips, 1919), 27 to 28 days (O. Wade, 1927), and 27.5 days (Johnson, 1931).

A number of factors have been known to modify the reproductive cycle of certain animals. Excessive fat is often a cause of sterility (Marshall and Peel, 1910). Captivity modifies the length of the cycle of many wild animals (Marshall, 1922). Hammond (1931) states that the breeding season of the rabbit in domestication in England has been prolonged. Fighting inhibited reproduction in cottontail rabbits (Dice, 1929). Food, locality, and length of season affected the Columbian ground squirrel in nature (Shaw, 1925), and weather conditions set the time of sexual activity of the thirteen-lined form (Drips, 1919). The shortening of the daily period of exposure to light from fifteen to nine hours almost prevents reproduction in the field mouse (Microtus agrestis). It is the female that is chiefly affected (Baker and Ranson, 1932).

Johnson and Wade (1931) found that a diet supposedly containing all the vitamins necessary for reproduction, ultra-violet radiations, ovarian implants, and ovarian extract injections caused no apparent effect on the genital functions of Citellus tridecemlineatus. They also found that anterior pituitary implants, with or without ultra-violet radiations, did not cause reproduction but did stimulate the uterus and follicles in the ovary to excessive growth and corpus luteum formation. Bissonnette (1932) treated anoestrous male and female ferrets with light from a 200-

watt incandescent electric light bulb for six to six and a half hours per night. He found that the females came on heat, copulated, and became pseudo-pregnant with mammary gland activity. In case of the males the results were less apparent, spermatogenesis being stimulated only to secondary spermatocyte and rare spermatid formation; no spermatozoa were found even at 71 days. It is well to note that he had only a few animals.

Hisaw (1927) states that a large amount of the food of the thirteen-lined ground squirrel consists of grasshoppers, and that they prefer these to grains.

Laboratory rodents do not give much information concerning reproduction in the ground squirrel. The guinea pig has an average cycle of 16 days (oestrus lasting about 24 hours) with corresponding changes in the ovaries, uterus, and vagina. These changes are accompanied by characteristic vaginal smears (Stockard and Papanicolaou, 1917, 1919; Selle, 1922). Bourg (1931) noticed histological changes in the genital tract of the mouse during the different stages of the oestrous cycle. E. Allen (1922) found the mouse to have a four to six day cycle with characteristic vaginal smears for each phase of the cycle, and Long and Evans (1922) found the rat to have an average four day cycle with characteristic vaginal smears, and histological changes over the entire genital tract. It might be noted that in all of

these cases the anoestrous period was of short duration. Hartman (1923) in working out the 28 day cycle of the opossum, and Evans and Cole (1928) the 48-60 day cycle of the dog, made use of the vaginal smear method.

The reproductive cycle of the ferret, a carnivore, seems to resemble that of the laboratory ground squirrel more than those of laboratory rodents do. The female has a long anoestrous period, the vulva enlarges at oestrus, ovulation occurs only after copulation, and oestrus is prolonged in the absence of pregnancy (Hammond and Marshall, 1930). These investigators also record much individual variation, some females giving birth to two litters in one season. Allanson (1932) observed the testes and accessory glands of the male ferret to be in full activity from March to July, followed by a period of regression during August, September, and October. The testes and epididymis enlarge during the active period, the diameter of the tubules showing similar cyclic changes.

Shih-Fan (1927) found a positive correlation between the length of the testis and the length of the body in the striped-hamster. Testes with a greater length than 11 mm. contained mature spermatozoa and showed active spermatogenesis. Thus the length of the testis was used to distinguish between immature and adult animals.

The functions of the gonads have long been a subject of experimental research. The hormone obtained from mature follicles (oestrin) has been shown to be responsible for the production of oestrus (Allen and Doisy, 1923; Marshall and Wood, 1923; Parkes and Bellerby, 1926; Papanicolaou and Blau, 1927; Parkes, 1930; Parkes, 1931; Doisy and Thayer, 1932).

M. Smith (1926) was able to interrupt pregnancy in the rat by the injection of follicular extract during the first five days of pregnancy. Oestrin, by its influence on the hypophysis, inhibits the development of the ovary in the immature rat (Leonard, Meyer, and Hisaw, 1931; Moore and Price, 1932). To the corpus luteum have been attributed various functions. That the corpus luteum hormone is necessary for implantation of the embryo has been verified (Corner, 1928; Courrier and Kehl, 1929). It also controls progestational changes in the uterus as shown by Parkes (1931) and Corner (1928). Pregnancy is maintained through the influence of the corpus luteum hormone (Harris and Pfiffner, 1929; Allen and Corner, 1930; Parkes, 1931; Johnson and Challans, 1932). Parkes and Bellerby (1927) inhibited oestrus with corpus luteum hormone (progestin), and Harris and Pfiffner (1929) found the injections of progestin interferred with parturition in the rat. Ovulation was also inhibited (Loeb, 1923). Nelson, Pfiffner,

and Haterius (1930) and Johnson and Challans (1932) by administration of corpus luteum extracts prolonged pregnancy by hours. According to the observations of Courrier and Kehl (1929) the corpus luteum apparently might cease functioning just before parturition. That the corpus luteum also is dependent on the secretions of the anterior pituitary has been shown by the administration of an alkaline extract of the latter (Evans and Long, 1922; Johnson and Sayles, 1929; Johnson and Hill, 1930; Hill and Parkes, 1930; Moore and Price, 1930). Follicular development and activity are in turn correlated with the secretions of the anterior lobe of the hypophysis as shown by implantation work (Smith, 1926, 1927; Smith and Engle, 1927; Zondek and Aschheim, 1927, 1928; Allen, 1928; Engle, 1928; Hartman, 1930; Claus, 1931; Johnson and Wade, 1931; Johnson and Foster, unpublished; and others).

Anterior pituitary implants in non-spayed females have caused superovulation followed by normal luteinization in mice and rats (Smith, 1926, 1927; Evans and Simpson, 1929; Hill and Parkes, 1931; and others). Superovulation was not produced in ground squirrels in this laboratory (Johnson and Wade, 1931; Johnson and Foster, unpublished) by implants of the anterior pituitary, but excess ovulation was obtained in mice by injections of an alkaline extract (Johnson and Hill, 1930).

The researches of Moore and Gallagher (1930) and of Moore, Price, and Gallagher (1930) have shown that the morphological and physiological activity of the vas deferens, Cowper's gland, seminal vesicle, and prostate in the male are maintained by a secretion from the testes. No effect was produced on the accessories of spayed females by injections of testicular extracts. Anterior pituitary implants do not seem to stimulate the testes as to growth and activity, to the extent that they do the ovaries (Smith and Engle, 1927; Hill and Parkes, 1930; Moore and Price, 1930; Johnson and Wade, 1931; Johnson and Foster, unpublished).

There seems to be a regulatory relationship between the hypophysis and the gonads (Moore and Price, 1932). Gonads function only when they are forcibly stimulated by certain secretions that are normally provided by hypophyseal activity. Hypophyseal activity, on the other hand, is to some degree controlled by gonadal secretions, for when these gonadal hormones are present in sufficient quantities hypophyseal activity is suppressed. These workers have also shown that sex-hormone antagonism does not exist as shown by simultaneous injections of testicular and follicular hormones in gonadless animals. The gonad-stimulating hormone of the anterior pituitary might be inhibited by the injection of either male or female hormones (Moore and Price, 1930; Meyer, Leonard, Hisaw, and Martin, 1930).

Besides stimulating the reproductive tract, the hormones of the anterior pituitary promote growth (Evans and Long, 1921; Evans and Simpson, 1926, 1927, 1931; Johnson and Sayles, 1929; Johnson and Hill, 1930; Smith, 1930) and may cause acromegaly (Teel and Cushing, 1930).

The luteinizing, growth-promoting, and gonadal-stimulating hormones of the anterior hypophysis have been successfully separated (Putnam, Teel, and Benedict, 1928; Teel, 1929; Cushing and Teel, 1930; Fevold, Hisaw, and Leonard, 1930, 1931).

The maturity-producing hormone of the anterior pituitary has also been extracted from the urine of pregnant women (Zondek and Aschheim, 1928; Evans and Simpson, 1931), but Evans and Simpson (1929) have shown that this is less effective than whole gland implants.

Some evidence has also been produced to show that the hormones of the anterior pituitary act through the ovary to produce mammary development (Corner, 1930; Evans and Simpson, 1931; Asdell, 1931; and others).

METHODS AND MATERIALS

General

The ground squirrels used for this study were obtained from west-central Kansas. They were of the variety Citellus

tridecemlineatus pallidus (Allen), or Citellus tridecemlineatus arenicola, the southern variety recently split off from pallidus by Howell (1928). The animals designated as laboratory animals had been in the laboratory since the preceding spring or longer.

The majority of the animals in the laboratory served as controls for the special experiments in most cases. Abnormally fat or thin animals were not included in any of the experiments. The animals occupied cages with wood bottoms measuring two by three feet. Wood shavings were used on the floor, and small wooden boxes in which the animals could build nests were usually provided. A few were kept in wire cages with tin bottoms. From one to eight animals were usually kept in one cage. The animals were well cared for, and mice and rats under similar conditions reproduced freely.

The temperature in the laboratory was maintained at about 70° to 80° F. by means of a steam heating system.

During the course of this study, most of the animals in the laboratory were observed weekly with few exceptions, and all sexual conditions were recorded. The following terminology was used to designate the sexual conditions of the animals: In case of the female when the vulva or external portion of the vagina was closed and no swelling was present, it was recorded as normal; when the vulva showed a slight swelling, as slightly swollen; a distinct swelling

such as occurs at heat, as swollen; and when the vagina opened, as open. In case of the male when the testes were small, redrawn in the abdomen, and no scrotum was present, the animal was recorded as normal; when the testes were slightly enlarged and beginning to descend and a scrotum beginning to form it was recorded as slightly scrotal; when the testes were large, and descended in a pigmented scrotum, it was recorded as scrotal.

Animals of both sexes were kept together during the entire year, thus giving them ample chance for reproduction.

Four groups each including four females and four males were respectively semi-starved, given a high protein diet, treated with ultra-violet radiations from two to six hours daily, and placed in a refrigerator from two to five weeks at a temperature of 2° to 6° C. in order to induce hibernation. A special control group (besides the general laboratory animals) was placed under similar conditions except for the treatments mentioned above.

Histological Technique

Tissues were obtained from male and female ground squirrels at short intervals between September, 1931, and August, 1932. Allen's modification of Bouin's fluid was used as a fixative almost universally for tissues of both sexes.

Hance's (1917) modification of Fleming's strong was tried in some cases for the fixation of testicular tissue, but this did not prove to be superior to Allen's modified Bouin. Kornhauser's (1930) hematin method with eosin-bluish as a counterstain was mostly used, although Heidenhain's iron hematoxylin gave good results for the staining of testicular tubules. Other stains were used experimentally, but were not found to be satisfactory.

Vaginal Smears

Vaginal smears were taken of laboratory females with open vaginae by means of a toothpick, the end of which was wrapped with absorbent cotton and dipped into Locke's physiological solution. This was then gently inserted into the vagina and the walls lightly scraped. By this procedure it was possible to obtain a sufficient number of cells to be characteristic of the smear, without any apparent pain to the animal.

For making permanent preparations of these smears fixation in warm modified Bouin's (Allen) for about 15 to 30 minutes was found to be very satisfactory. Hematin and eosin-bluish were used as these stained the cells almost instantly.

Implantation

Male and female animals which were inactive sexually were given implants of one rat pituitary per day, usually for seven to nine days. External genital conditions were recorded daily at the time of implantation. These experiments were conducted at intervals between September, 1931, and August, 1932.

Owing to the extreme variations in different individuals (especially the females) it was impossible to tell whether or not any two animals were in the same condition sexually. Thus some method had to be devised whereby each animal served as its own control.

The procedure used for the female was as follows: The animal was anaesthetized and about half of the right ovary removed. All genital conditions were recorded. After 24 to 48 hours had been allowed for recovery, the animal was implanted for four consecutive days. On the fifth day the remainder of the right ovary was removed with a piece of the uterine horn, and again all genital conditions recorded. After another 24 to 48 hour rest period, these were implanted usually for two to five days more. On the seventh to the eleventh day, depending upon the individual animal, the left ovary was removed with a piece of the left uterine horn. For the third time genital conditions were

recorded. Thus a series of three comparisons were available for each animal.

The males were treated in a similar manner except that the tunic of the right testis was simply pierced with a sharp needle and a few tubules gently forced out to provide a sample before implantation. Other animals of both sexes were treated in a similar manner with the exception that they were not implanted at all (but operated). This was done to see if removal of part of one gonad resulted in a hypertrophy of the remaining part, or of the other gonad, or both.

One assistant killed a rat, opened the skull dorsally, removed the gland wholly, and placed it in a small vial containing Locke's physiological solution heated to about body temperature. The gland was then picked up in a sterile glass canula. While a second assistant held the animal, the other made a small incision (usually on the leg) with a sharp needle, forced the canula along the dorsal side of the animal through the incision, and gently squirted the gland with a little physiological solution subcutaneously. The incision was then "sealed" with carbolated vaseline. The whole procedure required about two to three minutes, and no anaesthetic was used. All work was done under aseptic conditions in most cases.

SEXUAL CYCLE OF THE LABORATORY FEMALE

The Anoestrous Period (July to December, Inclusive)

The period from July to December, inclusive, might be said to be the anoestrous period of the laboratory female ground squirrel (Citellus tridecemlineatus) (Table I). The females were generally in good condition during this period, much fat being usually present in the mesenteries, mesovaria, and under the skin. A few animals had a slight tendency to become torpid in the late fall, but none of them went into actual hibernation. After the heat was turned on, all animals were again active and remained so through the winter.

During this sexually inactive period the vulva was very small and inconspicuous; its lips were fused so that copulation was impossible. Histological examinations showed the vaginal lumen to be small. The walls were lined with small epithelial cells of which only one to a few layers were present, and possessed a pinkish color. All cell layers, and especially the mucosa, were thin and gave an inactive appearance. The epithelial lining showed no cornification of the superficial layers (Fig. 5).

The average outside diameter of the uteri was found to be about 1.1 mm. These were pale pink in color, no congestion being present. The lumina were small; very little

Table I. Observations on the swelling of vulva and opening of the vagina in laboratory females[a]

Date	Total number females observed	Number females small vulva	Number females slightly swollen vulva	Number females swollen vulva	Number females open vagina
1930 July to Dec.	All[b]	All	0	0	0
1931 Jan. 9	29	28	0	1	0
24	28	16	0	10	2
Feb. 1	28	12	0	13	3
11	35(7)[c]	3	13	16(7)	3[d]
28	31(11)	0	10	15(11)	6[d]
Mar. 12	31(16)	3	5(4)	18(10)	5(2)
29	29(17)	1	4(3)	14(8)	10(6)
Apr. 10	29(10)	1	8	15(8)	5(2)
May 5	19(1)	12	6	1(1)	0
27	28	19	9	0	0
June 20	28	26	1	1	0
25	28(1)	26	0	0	2(1)
July 20	29	29	0	0	0
Sept. 14-18	13	13	0	0	0
22	13	13	0	0	0
Oct. 2- 5	13	12	0	0	1
12	12	11	1	0	1[e]
19	10	9	1	0	0
26-30	10	9	1	0	0
Nov. 4- 6	12	11	1	0	0
12	12	9	3	0	0
19	11	8	3	0	0
30	11	10	1	0	0
Dec. 6- 8	11	7	2	2	0
19	14(2)	12(2)	2	0	0
1932 Jan. 2	20(3)	14(3)	4	1	1
9	15(3)	12(3)	0	1	2
13-16	22(15)	16(11)	3(2)	2(1)	1(1)
21	27(11)	22(10)	1(1)	1	3
26-27	33(17)	25(13)	3(2)	2(1)	3(1)
Feb. 2-3	37(18)	29(11)	3(5)	2(1)	3(1)
7	25(18)	11(9)	7(7)	2(2)	5
13-14	41(19)	18(6)	10(8)	4(3)	9(2)
20	33(14)	18(5)	7(5)	1(2)	7(2)
27	33(14)	15(6)	5(4)	3(3)	10(1)
Mar. 5	32(12)	16(4)	5(4)	0	11(4)
12	42(10)	23(4)	7(2)	2	10(4)
19	35(9)	18(4)	5(1)	0	12(4)
26	37(10)	19(5)	6(1)	0	12(4)
Apr. 2	28(9)	14(5)	3	0	11(4)
9	29(11)	14(6)	4	1(1)	10(4)
16	26(10)	12(6)	5	1(1)	8(3)
22	26(8)	11(3)	3(1)	1(1)	11(3)
30	31(4)	11	6(1)	2(1)	12(2)
May 7	32(4)	13(2)	3	1(1)	15(1)[f]
14	25(4)	11(2)	3	3(1)	8(1)[f]
21	26(4)	11(2)	4	2	9(2)
28	27(5)	12(2)	5	1	9(3)
June 4	34(4)	18(2)	3	0	13(2)
11	31(4)	21(2)	3	0	7(2)
18	27(7)	15(6)	7	0	5(1)
25	29(1)	23(1)	2	0	4
July 2	25(1)	18(1)	2	0	5
9	27(1)	22(1)	1	0	4
16	30(2)	26(2)	2	0	2
23	32(2)	29(2)	1	0	2

[a] The observations up to September 14, 1931, were those of M. A. Foster.

[b] No genital development was seen in any handling of the animals nor in a number of special observations made on several animals at different times during this period.

[c] Numbers in parentheses on right indicate animals received as young in 1930 and 1931, respectively. Included in numbers to the left. Variations in the number of animals observed occur chiefly because some animals were used for experimental purposes.

[d] Two litters were born on February 28 from old females.

[e] Found dead on October 13.

[f] One litter born on May 10 from an old female.

secretion, if any, could be detected. Histological examinations here, too, revealed all cell layers to be thin and apparently inactive. The uterine glands, if visible at all, were small. In these, small or no lumina were present, and there was no secretion. The lining layer was almost cuboidal or very slightly columnar epithelium, the nuclei being at about the same level in all cells. The muscle layers were especially thin and very little contraction, if any, was noticed when the animals were autopsied. The blood supply was limited giving the uterus a pale color (Fig. 3).

The ovaries averaged 2.3 x 1.4 mm. in size. Eight females had ovaries of 2.0 x 1.5 mm., and one young female had an ovary measuring 2.0 x 1.0 mm., which is the smallest that we have on record. Microscopical examinations of these (Table II) over the entire anoestrous period revealed very compact bodies bounded by numerous oogonia. Follicles of varying sizes were usually present, the larger ones measuring .27-.30 mm. in their greater diameter. In nearly all cases young atretic follicles were also present. The interior portion of the ovary was almost filled with atretic follicles (Fig. 1). In some cases the ovary consisted almost exclusively of young atretic follicles.

Table II (A). Histological Observations On Laboratory Females

Date	Age	Size of ovary (mm.)	Size of uterus (mm.)	Condition of vagina	Size larger follicles (mm.)	Histological data[a]
1930						
July 15	Old	2.5 x 2.0	1.0		.34-.38	1,2,3,4,7
July 28	Old	Small	1.0	Closed	.30-.36	1,2,3,4,5
Aug. 7	Old	Small	Small	Closed	.20-.30	1,2,3,4
Aug. 8	Old	Small	Small	Closed	.30-.38	1,2,3,4
Aug. 8	Old	Small	Small	Closed	.30-.36	1,2,3,4
Aug. 8	Old	Small	Small	Closed	.34-.40	1,2,3,4,5
Sept.27	Old	2 x 1.5	1.0	Closed	.20-.34	1,2,3,4
Oct. 2	Old	2 x 1.5	1.0	Closed	.24-.30	1,2,4
Oct. 28	Young	2 x 1.5	1.0	Closed	.24-.30	1,2,3,4
Nov. 6	Old	2.5 x 2	1.5	Closed	.30-.40	1,2,3,4,5
Nov. 13	Old	2.5 x 2	1.5	Closed	.30-.40	1,2,3,4,5
Dec. 2	Old	2.5 x 2	2.0	Closed	.30-.40	1,2,3,4,5
Dec. 3	Young	2.5 x 2	2.0	Closed	.30-.40	1,2,3,4,5
Dec. 13	Old	2.0 x 2.0	2.0	Closed	.34-.44	1,2,3,4,5
1931						
Jan. 6	Young	2 x 1.5	1.0	Closed	.32-.36	1,2,3,4,6
Jan. 7	Old	2 x 1.5	1.0	Closed	.32-.38	1,2,3,4
Jan. 14	Old	2.5 x 1.3	1.1	Slightly enlarged	.36-.40	1,2,3,5
Jan. 14	Young	2.0 x 1.0	1.0	Closed	.36-.40	1,2,3,4,5,6
Jan. 14	Young	3.5 x 1.5	1.0	Closed	.34-.40	1,2,3,5,6
Jan. 16	Young	3.5 x 2.0	1.0	Closed	.34-.40	1,2,3,5
Jan. 26	Young	3.0 x 2.0	2.0	Closed	.36-.45	1,2,3,4,5
Feb. 14	Old	4.0 x 3.0	4.0	Open	.40-.45	1,2,3,4,5,7
Feb. 27	Old	3.0 x 2.0	1.5	Closed	.36-.40	1,2,3,4,5
Mar. 9	Old	3.0 x 2.0	2.5	Open	.30-.40	2,3,5
Mar. 31	Old	4.0 x 3.0	2.5	Closed	.36-.48	1,2,3,4,5,6,7
Apr. 14	Young	3.0 x 2.0	Pregnant[b]	Open	.26-.32	1,2,3,4,6,7
Apr. 14	Young	3.0 x 2.0	3.0	Slightly open	.30-.36	1,2,3,4
Apr. 21	Old	3.0 x 2.5	4.6[c]	Open	.36-.44	1,2,3,4,5,6,7
June 3	Old	2.0 x 1.5	1.5	Closed	.36-.40	1,2,3,4,5
June 3	Old	2.0 x 1.5	1.5	Closed	.30-.40	1,2,3,4,5,6
June 19	Old	3.0 x 2.0	1.0	Slightly enlarged	.26-.32	2,3,4
Sept. 8	Old	3.0 x 1.5	1.5	Closed	.17-.20	1,2
1932						
Jan. 30	Young	2.0 x 1.5	1.2	Closed	.30-.36	1,2,3,4,6
Feb. 9	Old	2.5 x 1.5	2.0	Open	.30-.38	1,2,3,4,5
Feb. 18	Old	2.5 x 1.5	.75	Closed	.20-.38	1,2,3,4
Mar. 8	Old	3.0 x 2.5	4.0	Open	.36-.64	1,2,3,4,5
Mar. 9	Old	2.0 x 1.5	.75	Closed	.32-.36	1,2,3,4,5
Mar. 9	Old	3.0 x 2.5	4.0	Open	.58-.60	1,2,3,4,5
Mar. 17	Old	2.0 x 1.5	.75	Closed	.32-.40	1,2,3,4,5
Mar. 18	Old	2.5 x 2.0	1.5	Closed	.22-.32	1,2,3,4
June 11	Old	3.5 x 2.0	1.0[d]	Closed	.20-.22	1,2,3,4,5

(B) Histological Observations on Field Females

1930						
June 20	Old			Open	.25-.30	1,2,3,4,7
Aug. 8	Old	Small	Small[e]	Closed	.25-.34	1,2,3,4,7
1931						
May 28	Old	3.0 x 2.0	2.5[f]	Slightly enlarged	.32-.36	1,2,3,4,5,7
1932						
May 6	Old	3.5 x 1.5	5.0	Open	.50-.54	1,2,3,4,5
May 6	Old	3.5 x 1.5	3.0	Open	.38-.40	1,2,3,4,5
May 7	Old	2.5 x 1.5	Pregnant	Open	.20-.22	1,2,3,4,7
May 21	Old	4.0 x 2.0	3.0[g]	Open	.36-.38	1,2,3,4,7
May 27	Old	2.5 x 2.0	2.0	Open	.64-1.80	1,2,5
May 31	Old	2.5 x 1.5	1.0	Closed	.30-.40	1,2,3,4,5
May 31	Old	2.5 x 1.5	1.0	Closed	.24-.42	1,2,3,4,5,6
May 31	Old	1.5 x 1.0	1.0	Closed	.26-.30	1,2,3,4
June 14	Old	3.5 x 2.0	1.0	Closed	.18-.20	1,2,3,4,6
June 19	Old	2.0 x 1.5	1.5	Closed	.20-.30	1,2,3,4

[a] The figures are used to show the structures present as follows: 1, oogonia; 2, atretic small follicles; 3, small follicles; 4, medium follicles (without cavities or with small cavities); 5, mature follicles (with cavaties); 6, medium large to large atretic follicles; 7, corpora lutea. The predominating stages are underlined once. Stages exceedingly abundant are underlined twice.

[b] Pregnant nineteen days.

[c] One day after parturition.

[d] Thirty-one days after parturition, but not lactating.

[e] Three months after parturition.

[f] Not lactating.

[g] Six days after parturition.

The description just given was typical of most of the anoestrous period except the latter part (late November and December) when a gradual hypertrophy of the entire genital tract began in a large number of the females.

The Period From January to June

The period from January to June generally included the period of apparent sexual activity (Tables I and II). There was a gradual universal hypertrophy of the genital tract accompanied by a decrease in visceral and subcutaneous fat. The five females at the end of Table III, showed no sexual activity during the entire period. The vulvae of three females showed slight swellings which did not culminate in the opening of the vaginae. One female (No. 1245) had two definite cycles as diagnosed by vaginal smears. One female had an open vagina before October 5, which was still open on October 13 when she was found dead in the cage. Of a total of ten to twelve females observed from October 12 to December 19, one had a slightly swollen vulva on October 12, and three on November 12. On January 2 one had an open vagina, and from then on the number of females with open vaginae gradually increased. Table III shows that many individual differences were exhibited among laboratory female ground squirrels.

Table III. Individual Sexual Observations on Laboratory Females

Fall, 1931, to Summer, 1932, Inclusive.

Animal number	Date observations were begun	Date of beginning and ending of vaginal enlargement	Days vagina enlarged	Date of beginning and ending of vaginal opening	Days vagina was open
1277	Sept. 18	Oct. 27-July 16	263±	Feb. 5-June 15	134±
1186	Oct. 13	Nov. 12-Jan. 12	61±	Never opened	0
1310	Oct. 19	Dec. 8-Feb. 17	71±	Dec. 12-Feb. 17	67±
1367	Jan. 13	Before Jan. 13-Feb. 17	35+	Jan. 16-Feb. 7	22±
1177	Jan. 23	Before Jan. 23-July 17	176+	Before Jan. 23-July 2	161+
1329	Dec. 19	Feb. 7-May 14	97±	Never opened	0
1117	Oct. 13	Feb. 7-May 10	93±	Feb. 10-May 10	90±
1265	Sept. 14	Feb. 7-April 26	79±	Feb. 10-Apr. 15	65±
1266	Sept. 14	Feb. 7-May 28	111±	Feb. 24-May 15	81±
1423	Dec. 20	Feb. 7-July 9	153±	Mar. 1-June 1	92±
1378	Jan. 13	Feb. 7-July 17	161±	May 21-July 2	42
1390*	Jan. 19	Feb. 7-May 7	90±	Feb. 29-Apr. 25	56
1356*	Feb. 11	Before Feb. 11-Mar. 12	30+	Never opened	0
957	Feb. 11	Before Feb. 11	36+	Feb. 16-Mar. 18 Open (Dead)	31+
1245	Feb. 11	Before Feb. 11-Mar. 28**	46+	Feb. 15-Mar. 27	41
		Before Apr. 22-July 20	89+	Apr. 22-July 15	84
1163	Jan. 19	Feb. 20	17+	Mar. 5-Mar. 8 (killed)	3+
1428*	Dec. 20	Mar. 19-June 8	81+	Mar. 23-June 5	74
X11*	Mar. 19	Before Mar. 19-June 8	81+	Mar. 20-June 6	78±
1320	Dec. 19	Mar. 23-June 1	70±	Mar. 26-May 10	45±
1283	Sept. 18	Apr. 19-July 16	88±	Apr. 22-June 18	57±
1250	Jan. 23	Apr. 26-July 23	88±	Apr. 30-July 19	80±
1286	Sept. 18	July 2-		July 12-	
1393	Jan. 23	Vagina not enlarged nor open by July 21, 1932.			0
1391	Feb. 14	Vagina not enlarged nor open by July 21, 1932.			0
X10*	Mar. 19	Vagina not enlarged nor open by July 21, 1932.			0
1273	Sept. 15	Vagina not enlarged nor open by July 21, 1932.			0
1278	Sept. 18	Vagina not enlarged nor open by July 21, 1932.			0

NOTATIONS - Females No. 1177 and 1250 had young in laboratory in 1931. All females were inactive sexually from dates of first observations to dates of beginning of vaginal swellings (if any), and from dates of ending of vaginal swellings (if any) to date of last observations.

(±) Since these females were observed weekly with few exceptions, the numbers given are approximately correct to ± 3.5 days. In other cases, observations were taken almost daily.

* Young, born in laboratory, 1931.

** No. 1245 had two cycles as indicated by dates.

From a histological standpoint it seemed necessary to consider three stages of development and regression during this period, namely: the enlarged vagina (pro-oestrum), the open vagina (oestrum), and the regression (metoestrum) stages.

The enlarged vagina stage was characterized by a hypertrophy of the entire genital apparatus. The vulvae ranged in size from a slight to a marked swelling which finally culminated, in the majority of cases, in the open vagina stage. All layers of the vagina as seen microscopically began to increase in thickness and the lumen gradually enlarged. Its walls were lined with large, nucleated epithelial cells which increased in numbers as prooestrum advanced. During the latter part of this stage the vaginal walls gradually became paler and drier, and the epithelial lining increased in thickness.

The uterus also showed a corresponding enlargement from the hypertrophy of all layers, and averaged about 3 mm. in outside diameter. The lumen also had increased in size. The uterine glands appeared as large, circular "nests" of cells in the mucosa. Along the latter part of the period, these increased in number and began to show lumina in which a whitish secretion could be detected. The cells immediately lining the uterine lumen had begun to elongate showing a seemingly active appearance. The blood supply of the uterus

had increased, resulting in a change of color from a pale pink to a deep pink.

The ovaries increased in size due primarily to follicular enlargement. The larger follicles of anoestrum now averaged about .34 to .40 mm. in diameter. Some of the oogonia of anoestrum had undergone transformation, a large number to atretic follicles, and the remainder to follicles of varying sizes from small to large. There seemed to be an increase of some follicles at the "expense" of others. The blood supply of the thecae evidently slightly increased as far as could be detected microscopically.

The open vagina stage evidently included late prooestrum, oestrum, and early metoestrum. All reproductive organs were at their maximum hypertrophy and gave an active appearance histologically.

The vaginal tissues had all become very thick, including the muscular, mucosa, and epithelial layers. The epithelial layers around the lumen became cornified. In many instances these cornified cells were seen sloughing off into the lumen which was now quite large. The walls of the vagina were white and dry (Fig. 6). In three females, vaginal "masses" consisting of large cuboidal epithelial and cornified cells held in clumps by a viscid whitish fluid was found protruding from the vagina. Probably these did not result from copulation as none of these females gave birth

to young, but were simply due to an excess accumulation and clumping of the cells mixed with secretion.

The uteri were large as compared to those of anoestrum. They averaged about 4 mm. in outside diameter. Their color had changed from a pink to a decided reddish color due to an intensive blood supply and congestion of the blood vessels. Every layer had hypertrophied to a great degree. The uterine glands of the mucosa were more numerous, larger, and all possessed lumina which were partially or totally filled with secretion. The lumen of the uterus was large and distended by much viscid, whitish secretion. The lining cells were now very columnar, their nuclei being at all levels in the cells, giving a pseudo-stratified appearance. During this period the muscular layers had increased tremendously, and the uterus possessed much power of contractility (Fig. 4).

The ovaries studied from females with open vaginae averaged 3.1 x 2.3 mm. as compared to 2.3 x 1.4 mm. during anoestrum. These contained from about two to twelve enlarged follicles which averaged approximately .37 to .48 mm. in diameter. Many small and medium follicles were present in a number of cases. Atretic young follicles were usually abundant. The blood supply of the ovary had increased as shown by the presence of large blood vessels in the thecae and stroma. The ovaries of this period gave the appearance

of mature ovaries, the larger follicles apparently being ready for ovulation.

The regression stage was marked by the vagina gradually losing its enlarged condition and eventually becoming closed. The cells of the cornified layers of the lining epithelium had completely sloughed off (in the late stage) and passed out in the lumen where they were apparently dissolved or digested by numerous leucocytes. Epithelial cells once more lined the lumen and the walls had again become pink, synchronously with leucocytic invasion. The mucosa, submucosa, and muscular layers gradually decreased in thickness.

The uterine layers also decreased in size simultaneously with vaginal regression. The glands were now reduced in number and size; the lining epithelial cells were once more assuming their almost cuboidal or low columnar form. The blood supply gradually decreased, causing the uterus to lose its reddish color.

The ovaries also decreased in size in a correlated manner with the vagina and uterus. This decrease in size was caused mainly by a decrease in the number and sizes of follicles. In the ovaries of at least four females mature follicles were seen which were becoming atretic. As to what became of the mature follicles that did not ovulate is not definitely known, but some of them did become atretic.

This regression stage just described in the vagina, uterus, and ovaries continued until the typical anoestrous condition was reached. The females had now become fat again.

Only one laboratory female (No. X8) gave birth to young on May 10. In spite of the fact that this female did not lactate (as she ate her young soon after birth), corpora lutea were still present thirty-one days after parturition.

The Results of Vaginal Smears

Throughout this paper the terminology proposed by Heape (1900), and adopted by Marshall and others, has been used. Anoestrous period or anoestrum, period of rest in the female; prooestrum, the first part of the sexual season; oestrus or oestrum, especial period of desire in the female; metoestrum, the short period when the activity of the generative tract subsides and the normal condition is resumed in case conception did not occur; and anoestrum the long period of rest in some animals. Such a cycle as the writer shall describe for the thirteen-lined ground squirrel consists of four periods: prooestrum, oestrum, metoestrum, and anoestroum. These constitute the anoestrous cycle.

The results obtained from vaginal smears taken from laboratory females with open vaginae follow:

Prooestrum. Characterized by the presence of large squamous nucleated epithelial cells (Fig. 7). In early prooestrum, some of the small epithelial cells and leucocytes of anoestrum persisted but as prooestrum advanced these disappeared. The large epithelial cells became very numerous and sloughed off from the walls into the lumen. One slight scraping of the vaginal walls might dislodge an exceedingly great number of these cells some of which came off in clumps. The nuclei stained a dark blue with hematin, and the cytoplasm a pinkish red with eosin. The vaginal walls gradually lost their pinkish color and became drier. Mucus secretion usually was present.

The development of the ovaries and uterus at this stage corresponded to that formerly described for the enlarged vagina stage.

Oestrum. The large epithelial cells of prooestrum apparently extruded their nuclei as in numerous cases cells which possessed eccentric nuclei could be detected. In a few instances some of these nuclei could be seen in the smear. At the peak of this stage, nothing but cornified cells could be found, and these were always very numerous. The cells sloughed off from the walls of the vagina in long, strand-like masses (Figs. 6 and 8), and in many instances

large masses of these cornified cells which stained red with eosin, were clumped, apparently held together by secretion. Leucocytes were never present at this stage. The walls were now dry and had changed from a light pink color in prooestrum to a whitish, "cheese-like" appearance in oestrum.

The conditions of the ovaries and uterus at this time corresponded to the stages described for the open vagina stage. The uterus contained much viscid, whitish secretion in its lumen.

Copulation supposedly occurs during this stage as found by Stockard and Papanicolaou (1917) for the guinea-pig and by Long and Evans (1922) for the rat. In one case in which a smear was taken from a female immediately after copulation, only cornified cells were present. These, plus an albuminous-like secretion, had formed a "plug" which stuck fast in the vagina. It is possible, however, that copulation could occur slightly earlier or later than this stage.

Metoestrum. Almost over night the vaginal lumen was invaded by a tremendous number of transitional and polymorphonuclear leucocytes. During oestrum some leucocytes could be seen below the epithelial lining of the uterus giving the appearance of a dark band beneath the surface. These apparently worked their way between the epithelial cells of the lining and invaded the lumen. As soon as the

leucocytes were present in the smear, the cornified cells began to decrease in number due probably to the digestive or dissolving power of these leucocytes. In many instances cornified cells could be seen with one to several leucocytes inclosed within their walls (Figs. 9 and 10). As metoestrum advanced the cornified cells became less and less numerous until they finally disappeared. The leucocytes also were greatly reduced in number, only a few remaining. The nuclei of some of these evidently fragmented forming what might be called polynuclear leucocytes. During the latter part of metoestrum, before the cornified cells had entirely disappeared, some few circular shaped epithelial cells with large nuclei were present in the smear. The vaginal walls were again moist and had resumed their former pinkish color. Some slight secretion was usually present.

As metoestrum gradually subsided anoestrum came on. At this stage it was very difficult to take smears; when it was possible to do so a few leucocytes together with a few large epithelial cells were present among small circular shaped epithelial cells with large nuclei (Fig. 11). These small epithelial cells formed the lining of the vagina at this stage, and were never obtained in as large quantities as the epithelial cells of prooestrum, the cornified cells of oestrum, or the numerous leucocytes of metoestrum in any smear. In some cases, however, these were found held

together in thin layers. The vaginal walls were now quite pink and usually a little secretion could be detected.

Because of the great amount of individual variation that was found to exist in the lengths of the cycles of these females (ranging from 17 to 263 days) no attempt was made to compute an average length of the cycle. Any such attempt would be almost futile and certainly very inaccurate and misleading.

It is important to bear in mind that the series of changes described for the vagina, uterus and ovaries are all correlated changes.

Two of the females of which smears were taken, became tame enough so that they could easily be handled without gloves.

SEXUAL CYCLE OF THE LABORATORY MALE

The Inactive Period

There was less individual variation among the male ground squirrels than in case of the females, from the standpoint of sexual activity. Most of the older males were inactive sexually from about the latter part of July to the earlier part of December. During this period the testes were small, averaging 11 x 7 x 5 mm. in size and were redrawn into the abdomen. No scrotum was present, and pigmentation was

entirely absent in the inguinal region. The animals were usually fat.

Histological observations revealed in practically all cases only spermatogonia and primary spermatocyte spiremes. The tubules usually possessed no lumina and averaged about 100 micra in diameter.

The Active Period

After this period of inactivity the testes gradually increased in size, a scrotum began to form, the hair gradually fell off, and the skin in that region became pigmented. This increase in size of the testes ultimately resulted in their complete descent into the now very pigmented scrotum, simultaneously with a decrease in the amount of fat present. The peak of sexual activity was during the month of April (Table IV).

During this stage of scrotality all stages of spermatogenesis were present as revealed by histological examinations. There seemed to be a slight tendency towards the greater production of spermatids and spermatozoa. That is, the primary and secondary spermatocytes seemed to decrease in number, while metamorphosing spermatids and spermatozoa predominated. The tubules averaged about 160 micra in diameter, and they now possessed lumina. In some cases the walls were very thin, and the metamorphosing spermatids

Table IV. Observations of Scrotality in Laboratory Males[a]

Date	Total number males observed	Number males normal[b]	Number males slightly scrotal	Number males scrotal
1930				
Sept. 1	20	20	0	0
Nov. 15			5	0
Dec. 1	21	10	6	5
1931				
Jan. 9	21	5	0	16
Jan. 24	31	3	0	28
Feb. 1	28	1	0	27
Feb. 11	32	0	1	31
Feb. 28	34	0	0	34
Mar. 12	31	0	0	31
Mar. 29	30	0	0	30
Apr. 10	30	0	0	30
May 27	30	3	5	22
June 17	30	12	6	12
July 1	30	20	7	3
July 20	28	26	2	0
Sept.22	9	9	0	0
Oct. 2-5	9	9	0	0
Oct. 12	9	9	0	0
Oct. 19	8	8	0	0
Oct.26-30	9	8	1	0
Nov. 4-6	12	9	3	0
Nov. 12	10	6	4	0
Nov. 19	10	7	3	0
Nov. 30	4	3	1	0
Dec. 6-8	15	14	1	0
Dec.19-20	12	11	1	0
1932				
Jan. 2	18	13	4	1
Jan. 9	15	11	4	0
Jan.13-16	22(10)[c]	10(4)	9(3)	3(3)
Jan. 21	30(6)	9(2)	10(2)	11(2)
Jan.26-27	63(11)	13(3)	35(4)	15(4)
Feb. 2-3	49(11)	12(2)	19(4)	18(5)
Feb. 7	(1)59(14)	(1) 7(3)	11(3)	41(8)
Feb.13-14	50(11)	5(2)	6(3)	39(6)
Feb. 20	45(10)	2(1)	9(3)	34(6)
Feb. 27	47(10)	1(1)	2(2)	44(7)
Mar. 5	(1)46(9)	(1) 1	3(1)	42(8)
Mar. 12	(5)59(10)	(5) 5(1)	1	53(9)
Mar. 19	45(11)	1(3)	4(2)	40(6)
Mar. 26	45(11)	3(3)	2(1)	40(7)
Apr. 2	31(6)	0(1)	9	22(5)
Apr. 9	37(8)	1(1)	13(3)	23(4)
Apr. 16	32(6)	1(1)	10(1)	21(4)
Apr. 22	30(4)	1(1)	11(1)	18(2)
Apr. 30	31(6)	4(3)	15(1)	12(2)
May 7	25(2)	2(1)	14(1)	9
May 14	23(2)	4(1)	9(1)	10
May 21	27(1)	7(1)	13	7
May 28	19(1)	6(1)	7	6
June 4	26(1)	8(1)	12	6
June 11	23(1)	6(1)	10	7
June 18	22(1)	5(1)	11	6
June 25	23(1)	5(1)	12	6
July 2	26(1)	11(1)	9	6
July 9	26(1)	12(1)	8	6
July 16	25(1)	16(1)	3	6
July 23	21(1)	13(1)	6	2

[a] Observations up to September 22, 1931, were done by M. A. Foster.

[b] By normal is meant that the testes are small and in the abdomen. The scrotum is not developed.

[c] Numbers in parentheses on right indicate number of animals received as young in 1931, and numbers in parentheses on left indicate number of animals just out of the refrigerator and, therefore, thin. All are included in the main number. Variations in number of animals observed was caused by the use of some animals for other experiments.

were embedded in the Sertoli cells around the periphery, while the spermatozoa were free in the lumen (Table V).

After this period of scrotality, the testes gradually decreased in size and were again redrawn into the abdomen. The scrotum finally disappeared, hair growing out in that region. Thus the males became sexually inactive again.

OBSERVATIONS ON NORMAL OUTDOOR ANIMALS

Females

The first outdoor animals were received from the field on March 31, 1932. Of this group of sixty animals only five were females. These females showed only very slight vaginal enlargements. They were thinner than the males received on the same date, indicating that they had come out of hibernation after the males. A second group of 44 animals was received on April 5. Of these nine were females, one of which showed no vulval enlargement , and two had slightly open vaginae. From this date on to April 20, when the last group was received, the number of females increased and most of these had enlarged or open vaginae (Table VI). The latest animals received were in better physical condition, showing that they had been out of hibernation for at least a few days.

Table V (A). Size and Development of Testes of Laboratory Males

Date	Size of testis (mm.)	Age	Stage of development[a]	Aver. size tubules micra
1930				
July 28	20 x 8	Old[d]	1,2,4,6,7,8	134
28	8 x 3	Old	1,2	100
Aug. 9	10 x 5	Old	1,2	140
9	10 x 6 x 6	Old	1,2	125
Sept. 23	7 x 6 x 5	Young	1,2[c]	86
27	12 x 7 x 6	Old	1,2[c]	114
28	14 x 8 x 7	Old	1,2[c]	90
Oct. 2	8 x 7 x 5	Young	1,2[c]	90
30	10 x 9 x 7	Young	1,2[c]	108
Dec. 6	13 x 6 x 5	Old	1,2,3,4[c]	116
6	13 x 8 x 6	Old	1,2,4[c]	120
20	14 x 7 x 5	Old	1,2,3,4[c]	130
29	17 x 7 x 5	Old	1,2,4,6,7,8[b]	160
1931				
Jan. 6	19 x 11 x 10	Young	1,2,3,4,6,7,8[b]	189
10	10 x 6 x 4	Young	1,2,4[c]	140
26	20 x 10 x 8	Old	1,2,6,7,8[b]	200
27	19 x 11 x 10	Old	1,2,6,7,8[b]	185
14	14 x 7 x 6	Young	1,2,4,6,7,8[b]	160
24	22 x 12 x 10	Old	1,2,4,6,7,8[b]	180
26	20 x 10 x 8	Old	1,2,3,4,5,6,7,8[b]	140
April 14	16 x 10 x 8	Old	1,2,6,7,8[b]	206
May 30	18 x 9 x 7	Old	1,2,6,7,8[b]	150
June 8	12 x 6 x 5	Old	1,2,6,7,8[b]	120
9	9 x 5 x 4	Old	1,2[c]	110
11	15 x 9 x 8	Old	1,2,6,7,8[b]	140
July 11	10 x 5 x 5	Old	1,2[c]	125
11	12 x 6 x 6	Old	1,2[c]	115
1932				
Jan. 30	17 x 8 x 8	Young	1,2,7	124
Feb. 18	10 x 5 x 4	Old	1,2	124
18	17 x 7 x 5	Old	1,2,3,4	156
March 29	17 x 9 x 8	Old	1,2,3,7	165
30	15 x 8 x 6	Old	1,2,3,6,7,8	144
30	10 x 5 x 4	Old	1,2,6,7,8	158
30	8 x 5 x 4	Young	1,2	84
30	11 x 4 x 4	Old	1,2	113
May 5	14 x 7 x 6	Young	1,2,3,6,7	136
5	10 x 5 x 4	Young	1,2	105
9	10 x 5 x 4	Young	1,2,3	98
9	10 x 5 x 3	Young	1,2	87
June 9	10 x 5 x 4	Young	1,2	90
20	10 x 5 x 5	Young	1,2	104

(B). Histological Observations on Field Males

1931				
April 14	16 x 9 x 7	Old	1,2,6,7,8[b]	160
14	17 x 10 x 8	Old	1,2,6,7,8[b]	170
1932				
May 17	10 x 8 x 7	Old	1,2,3,5,6,7,8	145
18	15 x 9 x 8	Old	1,2,6,7,8	144
18	15 x 7 x 7	Old	1,2,6,7	179
19	15 x 9 x 8	Old	1,2,6,7,8	206
19	16 x 8 x 8	Old	1,2,6,7	156
June 15	12 x 6 x 5	Old	1,2	88

[a] The figures are used to show the structures present as follows: 1, Spermatogonia; 2, Primary Spermatocyte Spiremes; 3, Primary Spermatocyte Mitoses; 4, Secondary Spermatocyte; 5, Secondary Spermatocyte Mitoses; 6, Spermatids; 7, Metamorphosing Spermatids; 8, Spermatozoa.
The predominating stages are underscored.

[b] Spermatozoa present in vas deferens.

[c] Spermatozoa absent in vas deferens.

[d] Old animals are animals that have been in the laboratory since the preceeding summer or longer. Young animals are animals born in the laboratory or received young from the field the preceeding summer.

Table VI. Observations on the Swelling of Vulva and Opening of Vagina in Field Females.

Date	Total number females observed	Number with small vulva	Number with slightly swollen vulva	Number with swollen vulva	Number with open vagina
March 31	5	0	5	0	0
April 2	5	0	4	1	0
April 5	9	1	6	0	2
April 9	20(6)[a]	1(0)	7(2)	6(2)	6(2)
April 14	77(58)	4(3)	16(11)	29(20)	28(24)
April 16	31(31)	0(0)	7(7)	11(11)	13(13)
April 22	99(11)	0(0)	17(4)	11(1)	71(6)
May 7	94	0	0	11	83
May 14	99	1	0	5	93[b]
May 21	90	3	0	5	82[c]
May 28	69	3	3	7	56
June 4	69	8	10	8	43
June 11	67	18	8	9	32
June 18	63	17	21	7	18
June 25	66	21	30	3	12
July 2	61	34	21	1	5
July 9	62	44	14	0	4
July 16	65	55	6	0	4
July 23	58	53	2	0	3

[a] Numbers in parentheses on right indicate number of animals received on that date. Included in number on left.

[b] Nine females were pregnant.

[c] Three females had young.

Of these females received from the field, at least four gave birth to young and one was pregnant when autopsied on May 7. Corpora lutea also were present. One female (No. 1172) was seen in copulation on April 22. In this case the gestation period was observed to be about twenty-eight days. The copulation dates as computed for the three other cases, using twenty-eight days as the gestation period, were one on April 17 and two on April 22. It is evident that most outdoor matings in 1932 took place between April 16 and April 23. Johnson and Foster (unpublished) found that most matings in 1931 were between April 14 and April 19, and four females in exceptionally good conditions copulated before April 14.

Referring back to the computed copulation date (April 12) of the one laboratory female it is seen that it was earlier than that of any field female. Comparisons of Tables I and VI show that the laboratory females were active sexually quite a while before the field females, but did not remain active sexually any appreciable time longer.

Ovaries studied from field females with open vaginae averaged 3.2 x 1.7 mm., the larger follicles averaging .30 to .41. One ovary contained follicles as large as .64 to 1.8 mm.

The uteri and vaginae resembled histologically those described for the laboratory females during the open vagina

stage. All layers were thick. The uterine glands were numerous and large, and the vaginal epithelium was cornified.

Males

Of the 55 males received on March 31, none was inactive sexually, eight were slightly scrotal and 47 were scrotal. They were all rather thin. The number of scrotal males increased as they were received later. The peak of the active season seemed to be in April (Table VII). The condition of these scrotal males resembled that of the laboratory males during the active period.

All stages of spermatogenesis were present, spermatids and spermatozoa predominating.

On July 16 about 8 per cent of these field males were still scrotal, while about 25 per cent of the laboratory males were scrotal. Thus the laboratory males remained scrotal to a later date than did the field males in 1932.

EFFECTS OF ANTERIOR PITUITARY IMPLANTS

Females

The administration of rat anterior pituitary implants to seven laboratory female ground squirrels for a period of five to nine days, caused a series of changes to appear in the genital tract.

Table VII. Observations of Scrotality in Field Males

Date	Total number males observed	Number males normal[a]	Number males slightly scrotal	Number males scrotal
March 31	55	0	8	47
April 2	53	0	11	42
April 9	104(13)[b]	2(0)	30(3)	72(10)
April 14	157(66)	0	25(19)	132(47)
April 16	27(27)	0(0)	8(8)	19(19)
April 22	169(7)	0(0)	1(1)	168(6)
May 7	140	3	34	103
May 14	157	4	12	141
May 21	135	6	34	95
May 28	101	5	47	49
June 4	104	20	42	42
June 11	80	19	29	32
June 18	76	20	20	36
June 25	65	17	26	22
July 2	67	33	16	18
July 9	73	37	21	15
July 16	61	43	9	9
July 23	67	48	12	7

[a] By normal is meant that the testes are small and in the abdomen. The scrotum is not developed.

[b] Numbers in parentheses on right indicate animals received on that date. Included in number on left.

External effects were marked by an enlargement of the vulva, which finally culminated in the opening of the vagina. The number of implants required to "bring" any animal to a certain degree of development, depended upon that individual animal and also upon the season of the year. During the months of September, October, and November an average of five implants was necessary to produce a slight enlargement of the vulva and an average of seven to produce an open vagina. In December, January, and February when the sexually active season was approaching, only two implants were necessary to produce a slight vulval enlargement; and four, an open vagina. The vagina could be kept open by continued implantation as evidenced by female No. 1389 which received twenty implants.

Histological observations of the vagina during the open stage, revealed all the changes accompanied by oestrum. All layers hypertrophied, the epithelial lining became thick, and the outer layers cornified, the cells sloughing off into the lumen. In the smears, the leucocytes and small epithelial cells of anoestrum, were replaced by the large epithelial cells of prooestrum, and these in turn by the cornified cells of oestrum.

The uteri also increased in size from a hypertrophy of all layers, measuring 3 to 4 mm. in outside diameter. That of a female receiving twenty implants reached 5 mm. in

diameter. The blood supply was very much increased, which resulted in a congestion, giving the uteri a deep reddish color. Histological examination of the uteri showed all layers to be thick. Numerous uterine glands were usually present which possessed lumina filled with secretion in most cases. The lining epithelial layer became columnar giving the same pseudo-stratified appearance of oestrum. Five additional females for which no histological data were available, showed increased sizes of the uteri and vaginae.

The ovaries increased tremendously in size as a result of pituitary implantation, becoming of the "mulberry" type in many cases, from the distention of the numerous very large follicles. Some of the larger follicles reached a size of 2 mm. in their greatest diameter. A great number of them exceeded 1 mm. in diameter. The blood vessels of the thecae became congested, and finally extravasated blood causing the follicles to become hemorrhagic in many instances. Usually after six to eight implants luteinization began. The follicles in most cases became hemorrhagic and the lutein cells seemed to surround the blood in the follicular cavity, sometimes "entrapping" the ovum.

Johnson and Wade (1931) and Johnson and Foster (unpublished) found the same results after anterior pituitary implants in the ground squirrels.

Figure 12 shows a section through a piece of the right ovary of female No. 1430 before implantation; Figure 13, a section through the remainder of the right ovary of the same animal after four implants; and Figure 14, a section through the left ovary of the same animal after nine implants.

Males

The effects produced in nine males were by no means so apparent as in the females, when implanted for a corresponding length of time. Externally, the effect was not very pronounced. The testes did not enlarge to the extent that they did during the "scrotal" period, nor did they descend as fully. In some cases, a slight degree of enlargement could be detected after about the fifth implant.

Histological examinations, however, revealed rather definite changes. Mitoses of primary and secondary spermatocytes were quite numerous as compared to the control piece of the testes before implantation. Spermatogenesis was stimulated to the formation of secondary spermatocytes and spermatids. Many of the spermatids were in the metamorphosing stage. Only in one case were spermatozoa found, and these could be accounted for. The control piece before implantation contained some metamorphosing spermatids, and these had apparently been stimulated to the formation of spermatozoa.

After four implants there seemed to be a smaller number of primary spermatocyte spiremes than in the control piece before implantation. In most cases, after four implants, mitoses of the first maturation division were present, and in one case mitoses of the second maturation division. After eight to nine implants more mitoses of both maturation divisions were present, more secondary spermatocytes, spermatids, and metamorphosing spermatids.

The tubules averaged about 100 micra in diameter before implantation, about 120 micra after four implants, and about 137 micra after eight to nine implants. The measurement for the four implants is probably a little too high as the tissues of two animals were not available. Probably about 115 micra would be more correct.

The implanted females showed all the characteristics of the animals in heat, but no increased desire for copulation. The males showed no increased sexual excitement. Reproduction did not occur.

Implantation did not seem to hinder the animals, since they were in a healthy condition during and after implantation.

In hibernation work (not reported) some animals were implanted with brain tissue, to serve as controls for anterior pituitary implanted animals. No precocious sexual development resulted from these brain implants.

Four males and four females were treated in a way similar to the implanted animals, except that they were not implanted. These showed no appreciable hypertrophy of the gonads resulting from partial removal or total removal of one gonad, at least not during the time required for the experiment.

THE EFFECT OF VARIOUS CONTROLLABLE LABORATORY FACTORS

Semi-starvation

Since fatness was found to be a cause of sterility (Marshall and Peel, 1910), it was thought possibly that semi-starvation might have some effect on reproduction in the ground squirrel. With this in mind a group of four males and four females were semi-starved as indicated by the loss of body weight. These animals were weighed and observed as to their sexual conditions every week. The males lost 26 per cent and the females 20 per cent of their original body weights. Careful comparisons of these animals with their controls showed no advanced sexual development.

High Protein Diet

Hisaw and Emery (1927) found that grasshoppers were an important source of food supply for the thirteen-lined ground squirrel. They also state that they prefer grass-

hoppers to grains. It was noted in this laboratory that young ground squirrels grew at a faster rate when fed milk after they were weaned.

A second group of four males and four females were fed grasshoppers for about two weeks, and rat flesh for the remainder of the time to see if this high protein diet would have any influence on reproduction. This, of course, was added to the regular food given the general laboratory supply of animals. The animals preferred the rat flesh to feed as indicated by the fact that very little feed was eaten until all of the rat flesh had been consumed.

After weekly observations, no sexual advancement could be detected as compared to the control group under similar conditions but not fed rat flesh.

Ultra-Violet Radiations

It is generally thought that sunshine is necessary for normal reproduction in most animals. With this as a hypothesis, a third group of four males and four females was treated with ultra-violet radiations of two to five hours daily from December 19, 1931, to July 1, 1932. The animals were kept in open wire cages where light could easily penetrate. The apparatus used was a mercury arc ultra-violet lamp. This lamp transmits slightly more ultra-violet light than is found in sunshine, and according to

Dr. J. S. Hughes of the Chemistry Department of this College will cure rickets in chickens in half-hour radiations.

Again careful weekly observations revealed no sexual advancement over the control group under similar conditions minus the radiations.

It might be added that the thirteen-lined ground squirrels in nature reproduce soon after they emerge from hibernation in the spring, before they have a chance of getting much access to sunlight.

Hibernation

In nature these animals normally go through a period of hibernation during the winter. Since they do not hibernate in our laboratory, it was thought that hibernation might be one of the prerequisites for normal reproduction.

A fourth group of four males and four females used in hibernation studies (not reported) were taken back to the refrigerator for a period of two to five weeks. Female Number 1117 hibernated four days and was partly torpid three days, and males No. 1293 and X35 were in hibernation four days. Male No. 1375 and females No. 1310 and 1405 hibernated for a period of three days each. This work was done during the latter part of October when hibernation in the field would normally occur.

Weekly sexual observations showed no significant genital effect on these animals. Whether or not hibernation tends to retard sexual activity in the spring is not known. In the experiment just mentioned, the treated animals came into sexual activity at about the same time that the majority of laboratory animals did. Female No. 1186 had a slightly enlarged vulva as early as November 19; female No. 1310 had an enlarged vulva on December 8; female No. 1117 a slightly enlarged vulva on February 7; and female No. 1405 a slightly enlarged vulva on April 2.

Males No. X35 and 1375 were slightly scrotal on December 8 and December 19, respectively; male No. 1293 was slightly scrotal on January 15; and male No. 996 died on November 2, before it showed any active sexual development.

These animals had not been in actual hibernation (even though they were in the refrigerator) long enough to make any very definite statement about the effect of hibernation.

However, the results of these experiments seem to indicate that there might be some other factors, besides those tried here, that are prerequisites for normal reproduction in the thirteen-lined ground squirrel.

DISCUSSION

The early activity of the laboratory animals, and the lack of normal reproduction were especially striking. It

is apparent that the ground squirrels in nature are subjected to some factors that are inhibited or absent in captivity. After this study the cause of this lack of reproduction is not yet known, but it is hoped that the results will give some insight to a successful attack for the solution of this problem.

Stockard and Papanicolaou (1917) state that in the guinea-pig the development and degeneration of the uterine and vaginal tissues are coincident with the development and degeneration of corpora lutea. Many ground squirrels in which histological development and degeneration of the tissues were manifested, did not possess corpora lutea at any time during the year. Therefore, in the ground squirrel at least, corpora lutea are not entirely responsible for the development and degeneration stages that the uterus and vagina go through during the sexual cycle. However, progestin might tend to keep up these processes longer after oestrin has played its part in sensitization of the tissues.

In one ovary which possessed normal looking corpora lutea, several large follicles were present. In this case it seemed that the corpora lutea had not prevented follicular development.

Another striking feature was the almost persistent number of atretic small follicles present throughout the

year. In several cases the ovaries were found to consist almost entirely of these atretic follicles. Some few mature follicles were also found which seemed to be in a degenerate condition. The cause of this atresia and the manner in which it takes place is not definitely known. Some small follicles seemed to keratinize while with some of the larger ones follicular cells which possessed pycnotic nuclei, seemed to be invariably present. These cells broke away from the walls of the follicle and in some cases remained attached to the ovum as it was resorbed or degenerated. In other cases the ovum was free in the cavity. More work will have to be done on this phase of the work, however, before a satisfactory explanation of this atresia can be put forth.

Bissonnette (1932) produced oestrus, copulation, and pseudo-pregnancy in three anoestrous female ferrets by treatments of six to six and a half hours per night with an ordinary 200 watt incandescent electric light bulb. His study, however, included only three animals, which are too few from which to draw very strong conclusions. Of course, the ground squirrels might not be affected by light to the extent that ferrets are, and his results might be verified with a greater number of animals.

SUMMARY AND CONCLUSIONS

1. The anoestrous period of the laboratory female ground squirrel generally extended from the earlier part of July to the latter part of December. All genital organs were in an inactive histological condition.

The period when sexual activity may occur extends from January to June. At this time the organs may be hypertrophied giving an active appearance. Much individual variation among females occurred. Only one case of reproduction in the laboratory was recorded.

2. Vaginal smears showed: (a) Prooestrum to be characterized by the presence of large, nucleated squamous epithelial cells; (b) Oestrum, by the presence of numerous cornified cells; (c) Metoestrum, by the presence of many leucocytes among the few remaining cornified cells; and (d) Anoestrum, by the presence of a few leucocytes and small epithelial cells.

3. The inactive period of the laboratory males extended generally from July to December. In most cases only spermatogonia and primary spermatocyte spiremes were present. The tubules average 100 micra in diameter.

The active period extended from January to June. All stages of spermatogenesis were present during this period, spermatids and spermatozoa predominating. The

tubules averaged 160 micra in diameter.

4. Laboratory males and females became sexually active before the field animals, and the laboratory males had a slight tendency to remain active a little later.

5. Pituitary implants in the females produced follicular enlargement and in some cases luteinization, but not superovulation nor reproduction. The entire genital tract was caused to hypertrophy.

In the males the effect was less pronounced. Spermatogenesis was stimulated only to secondary spermatocyte and spermatid formation.

6. Semi-starvation, a high protein diet, ultra-violet radiations of two to five hours daily, and hibernation did not cause advanced sexual development nor reproduction in the thirteen-lined ground squirrel.

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EXPLANATION OF FIGURES

Fig. 1. A section through the right ovary of ground squirrel No. 1164 in anoestrum. X28. The larger follicles measure about .32 mm. in their mean diameter.

Fig. 2. A section through the right ovary of ground squirrel No. 1143 in oestrum. X18. The larger follicles measure about .5 mm. in their mean diameter.

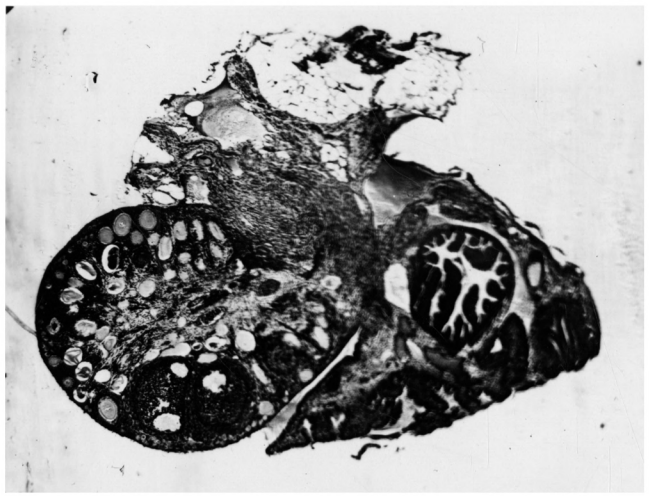

Figure 1.

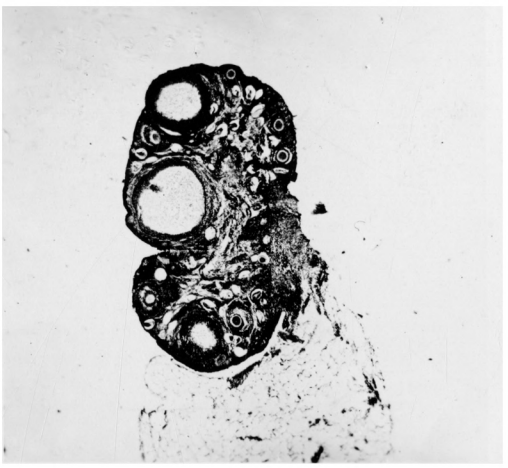

Figure 2 .

Fig. 3. A section through the uterus of ground squirrel No. 1164 in anoestrum. X32. Note especially the few, small uterine glands.

Fig. 4. A section through the uterus of ground squirrel No. 1143 in oestrum. X32. The glands are numerous and possess large lumina.

Figure 3.

Figure 4.

Fig. 5. A section through the vagina of ground squirrel No. 1089 in anoestrum. X28. No cornification of the lining epithelium is present.

Fig. 6. A section through the vaginal walls of ground squirrel No. 1143 in oestrum. X28. Note the presence of epithelial cornification. Some cells are seen in the process of sloughing off.

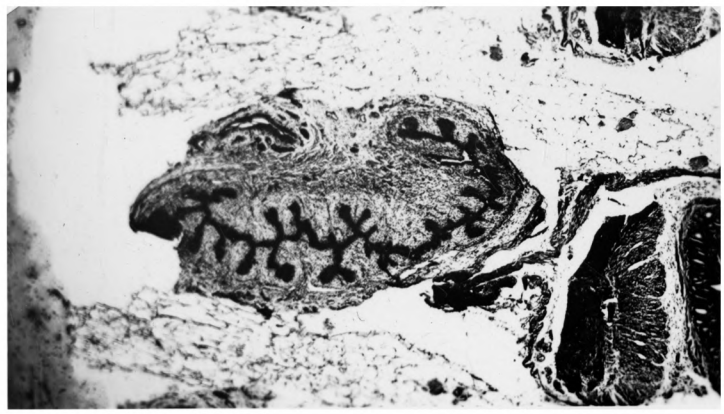

Figure 5.

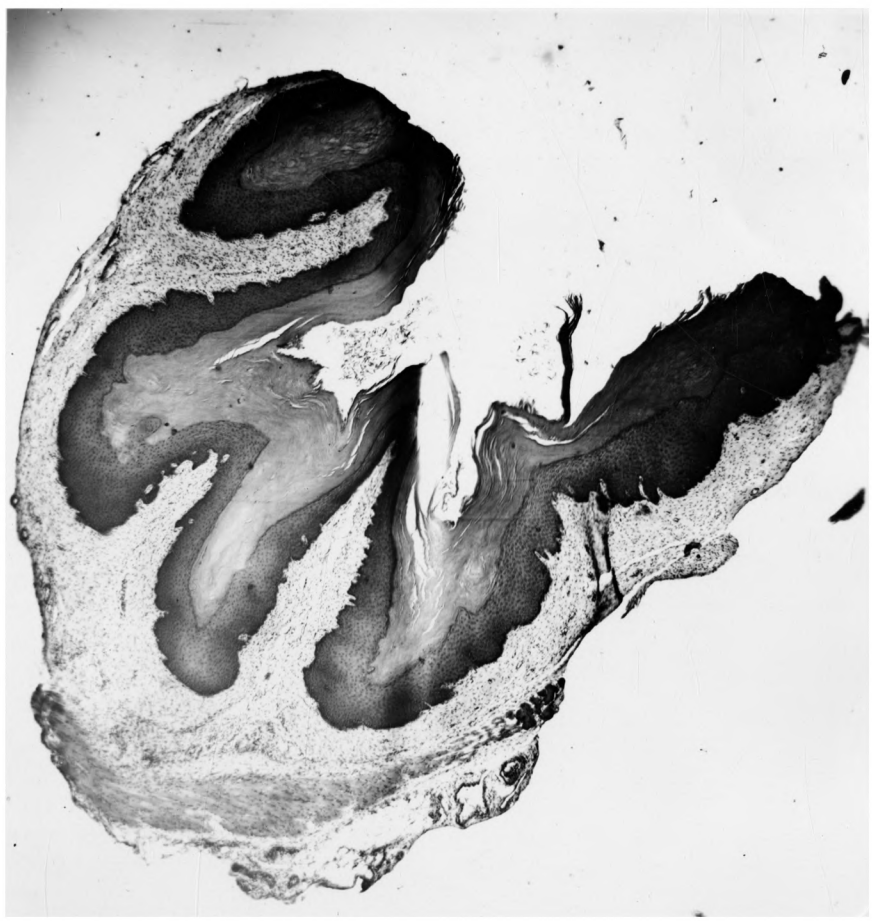

Figure 6.

Fig. 7. A photomicrograph from the vaginal smear of ground squirrel No. X11, showing the nucleated epithelial cells of prooestrum. X1000.

Fig. 8. A photomicrograph from the vaginal smear of ground squirrel No. 1390, showing the cornified cells of oestrum. X1000.

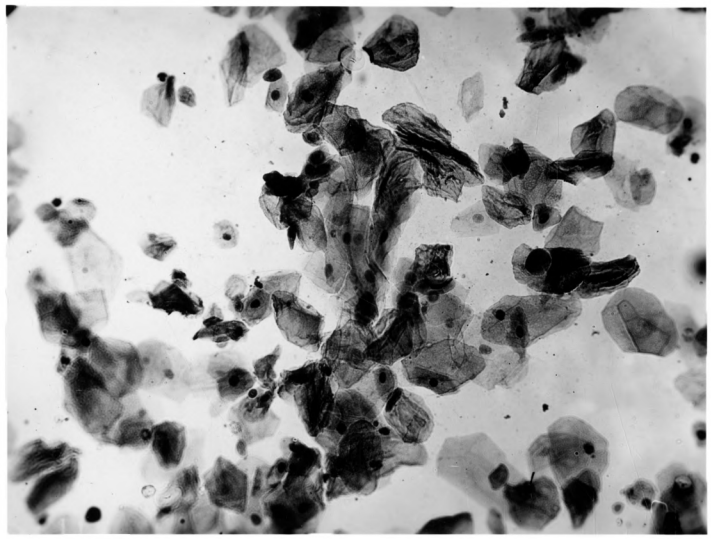

Figure 7.

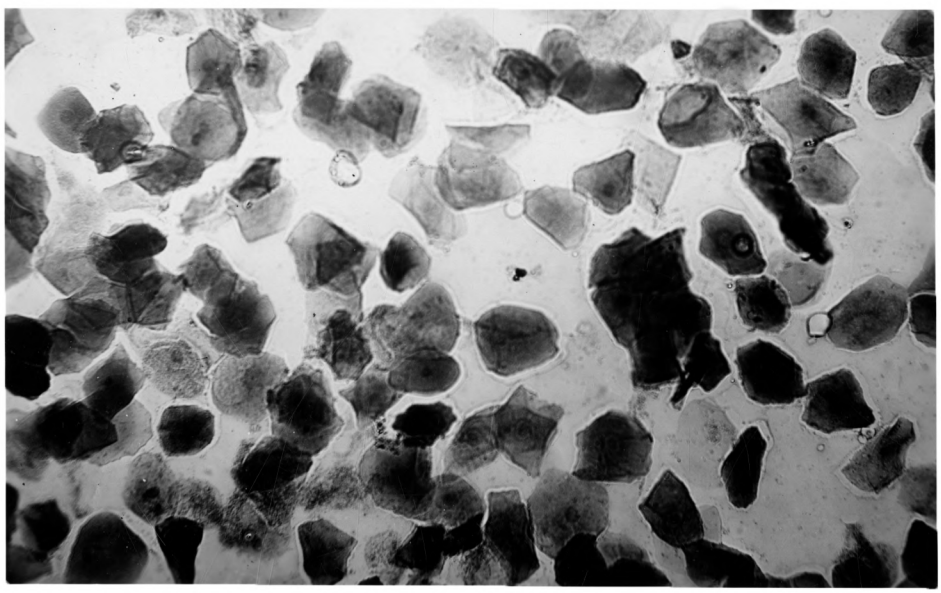

Figure 8.

Fig. 9. A photomicrograph from the vaginal smear of ground squirrel No. 1177, showing the numerous leucocytes and few cornified cells of metoestrum. X1000.

Fig. 10. Same as Figure 9. X4000.

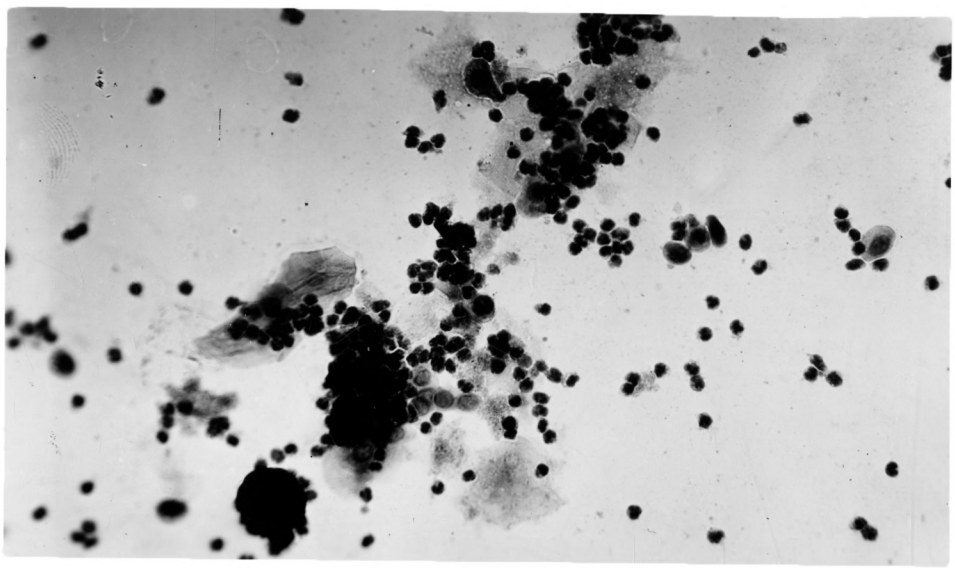

Figure 9.

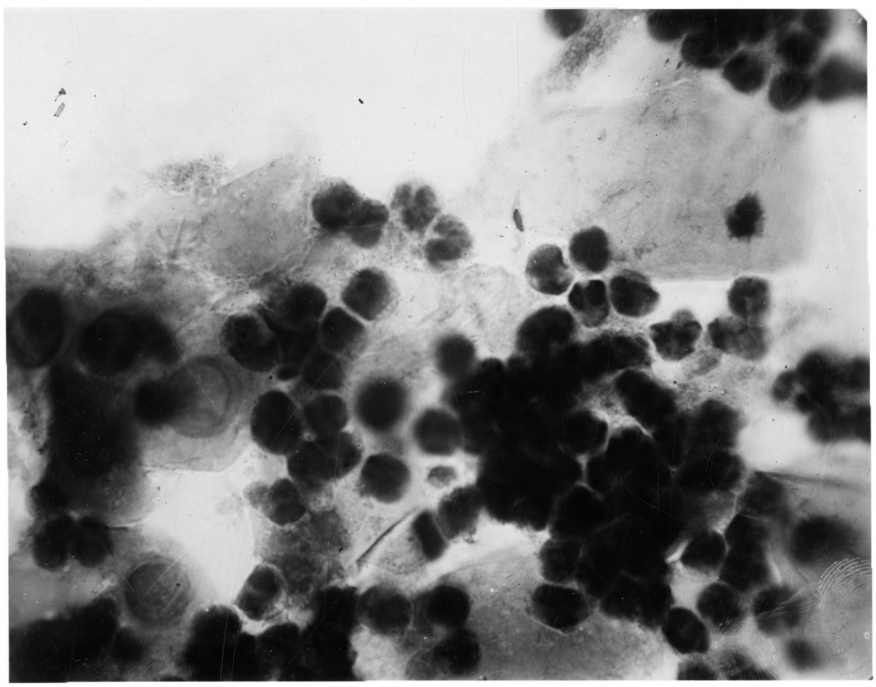

Figure 10.

Fig. 11. A photomicrograph from the vaginal smear of ground squirrel No. 1245, showing the leucocytes and small epithelial cells of anoestrum. X1000.

Fig. 12. A section through a portion of the right ovary of ground squirrel No. 1430 before receiving any implants. X20. The larger follicles measure about .34 mm. in mean diameter.

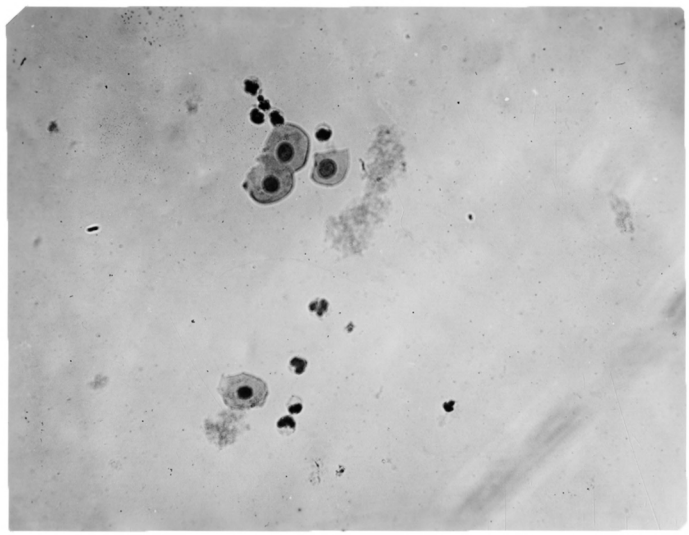

Figure 11.

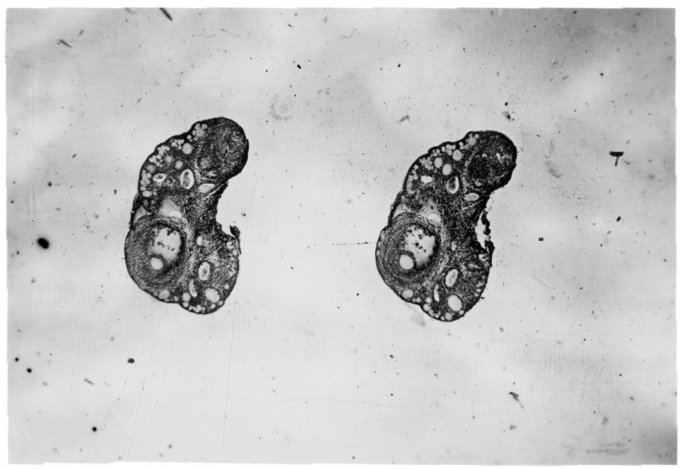

Figure 12.

Fig. 13. A section through the remainder of the right ovary of ground squirrel No. 1430, after receiving four daily implants of rat pituitaries. X20. The larger follicles measure about .56 mm. in mean diameter.

Fig. 14. A section through the left ovary of ground squirrel No. 1430 after eight daily implants of rat pituitaries. X20. The larger follicles now measure about 2.0 mm. in mean diameter.

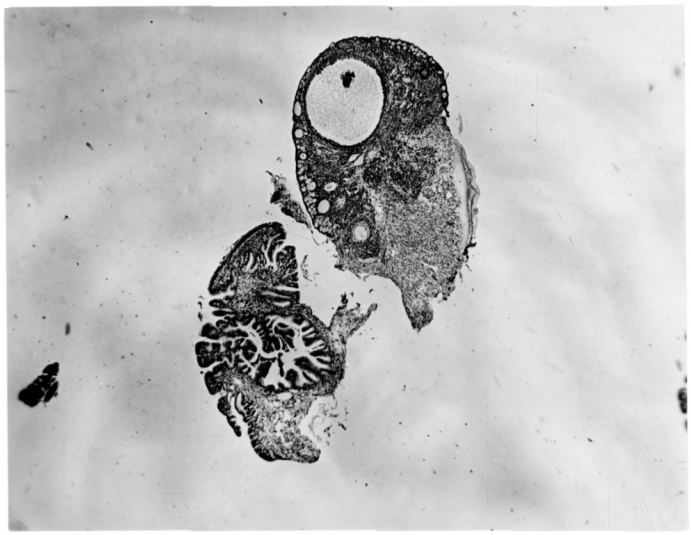

Figure 13.

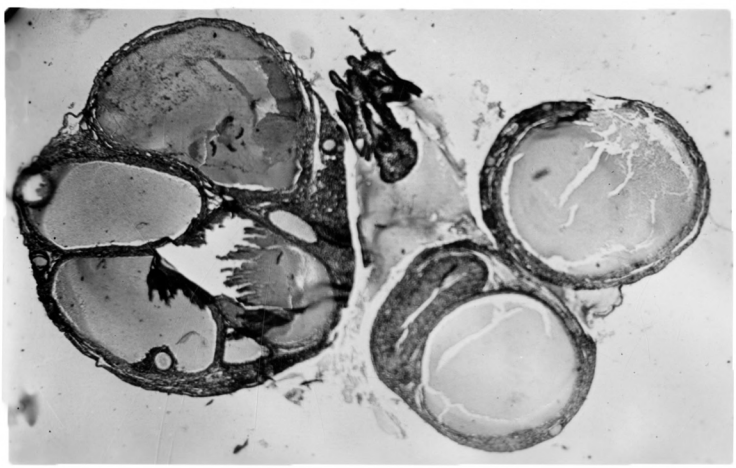

Figure 14.