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Circannual Changes in Serum Testosterone Concentrations of Adult and Yearling Woodchucks (*Marmota monax*)[1]

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ABSTRACT

Testicular volumes and serum testosterone concentrations were determined biweekly in 5 adult and 4 yearling woodchucks maintained indoors from December through August. Food and water were provided ad libitum except for 2 mo beginning at the winter solstice when feeding and lighting (12L:12D) supplemental to available natural light were discontinued. Temperatures fluctuated with outdoor temperatures ≥ 4°C. No significant hibernation occurred. Testes in adults were small in December (0.3–1.8 cm^3), largest in February and March (3.5–5.6 cm^3), and smallest in late June (0.1–0.5 cm^3). Testosterone was basal in December (<0.6 ng/ml), maximal (3.4–6.6 ng/ml) between early January and late March, and minimal from April through August (<0.8 ng/ml). In yearlings, maximum testes volumes (1.6 cm^3) and serum testosterone (0.9 ± 0.2 ng/ml) were less, and occurred later, than in adults. Testosterone levels and testis volumes measured in newly captured woodchucks in March and April and again 2–3 mo later were generally similar to those of their laboratory counterparts.

Thus, in woodchucks: 1) annual cycles of testosterone production and of testes recrudescence and regression parallel each other with maxima during the short, late-winter breeding season; 2) those cycles are not altered significantly by the absence of hibernation or the present conditions of captivity; and 3) yearling males apparently are not an important part of the breeding population.

INTRODUCTION

The woodchuck (*Marmota monax*), a North American hibernating sciurid rodent, is used as a model for several human conditions, including chronic viral hepatitis and subsequent development of primary hepatocellular carcinoma (Summers et al., 1978; Young and Sims, 1979). Fertility in laboratory-maintained captive woodchucks is low (Hamilton, 1934; Grizzell, 1955; Young and Sims, 1979; Concannon et al., 1984), and information on their reproductive biology is limited (Concannon et al., 1983). Woodchucks have one short breeding season a year in late winter following emergence from hibernation. The testes of adults are regressed and abdominal prior to hibernation in early autumn and testicular recrudescence occurs during hibernation.

In the vicinity of Ithaca, New York (42°30′N, 76°30′W), females give birth to their single litter during the first 3 wk of April following a 31–32-day gestation period (Concannon et al., 1983). Matings occur during the first 3 wk in March as females are emerging from hibernation. Adult males emerge from hibernation 1–3 wk earlier with large, scrotal testes containing mature sperm (Rasmussen, 1918; Hamilton, 1934). At emergence, degenerative changes in the spermatogenic epithelium were already evident; spermatogenic activity progressively decreased throughout the breeding season, and the testes were devoid of mature spermatozoa by the end of April (Christian et al., 1972). The interstitial cells have been reported to increase in both size and number as the seminiferous

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tubules decrease in size (Rasmussen, 1917). Christian et al. (1972) noted a concurrent increase in testis weight during May, as an interruption in an otherwise steady decline in weight from February through September, and noted an increase in the ratio of interstitial tissue to tubules. The apparent growth of interstitial tissue following the breeding season and during the period when spermatogenesis has been suspended is paradoxical. Testosterone levels in wild or captive males have not been previously reported.

The present study was conducted to determine changes in serum testosterone levels and testis size in individual laboratory-maintained adult and yearling captive woodchucks before, during, and after the expected breeding season, and to relate the findings to those observed in recently captured animals in an attempt to ascertain whether the conditions of captivity had altered the normal annual cycle.

MATERIALS AND METHODS

Colony Animals

Five adult and 5 juvenile (recently weaned) male woodchucks were captured locally in box traps between May and September. They were maintained outdoors in chain link runs with nest boxes until December 8, and were then housed indoors in individual stainless steel cages (0.7–0.9 m^2) each containing a 0.07–0.04 m^2 wood or metal nest box and straw for bedding. Available natural light was supplemented with incandescent white light on a schedule of 12L: 12D. Minimum temperature was maintained above 4°C by thermostatically controlled electric heaters, and maximum temperature fluctuated with outside ambient temperature (Concannon et al., 1984). Animals were provided with commercial rabbit pellets and water ad libitum until December 21, when a 9-wk period of conditions considered conducive to hibernation was initiated. During this period, food and water were removed, available ambient light was no longer supplemented with incandescent light, and the animals were checked weekly for hibernation status. A woodchuck was considered to be hibernating if it was curled in a ball, its eyes were closed, its body was cool, and it responded only minimally when handled.

Adult females were pair-housed with the adult males, starting December 21 through February 21, until the females were confirmed pregnant (n=2) or for the duration of the study (n=3). Juveniles (referred to hereafter as yearlings) were housed individually in the same room as the adults for the duration of the study. One of the yearlings died during the period when food was unavailable, and only results for the remaining 4 yearlings are reported.

Blood Collection and Testosterone Assays

Blood samples were collected at biweekly intervals from December 8 until August 5. Additional samples were obtained 24 h apart to assess daily variations in serum testosterone concentrations. Such samples were obtained from 3 of the adults in early February, from 4 adults in late February, and from 7 additional adults during February in another colony in which the housing conditions were comparable and testis development obvious. Samples were collected by femoral venipuncture into evacuated tubes following anesthesia induced by intramuscular injection of 1.5–3 ml of a solution containing xylazine (7 mg/ml) and ketamine hydrochloride (70 mg/ml). Blood was allowed to clot 8–18 h at 5°C prior to centrifugation; serum aliquots were stored at −20°C until assayed.

Testosterone levels were determined by radioimmunoassay (Ismail et al., 1972). Approximately 1200 cpm of [1,2,6,7-^{3}H] testosterone were added to 500 μl of serum to monitor loss of testosterone during extraction. Each sample was extracted by shaking with 5 ml of hexane:benzene (2:1, v/v) for 2 min, and the extract was partitioned against 1 ml of distilled water. Separations were accomplished by decantation following freezing in a dry ice-acetone bath. Each sample was reextracted and the combined extracts evaporated to dryness at 40°C. The total extract was equilibrated for 18–24 h at 5°C with 1.0 ml of the assay buffer. An aliquot (300 μl) was removed for determination of extraction efficiency and duplicate 300-μl aliquots were used for assay. The assay buffer was 0.01 M phosphate-buffered saline (PBS) containing 0.01 M EDTA, 0.1% gelatin, and sodium azide. Specific reagents included rabbit antiserum against testosterone-11-succinyl-bovine serum albumin and [^{125}I]-labeled testosterone-11-succinyl tyrosine methyl ester (Micromedic Systems, Inc., Horsham, PA). Relative cross-reactivity to the antiserum was 100%, 31%, and 15% for testosterone, dihydrotestosterone, and 19-nortestosterone, respectively and <1% for other androgens.

The duplicate 300-μl extracts were each incubated with 50 μl testosterone antiserum, 100 μl of [^{125}I]-labeled testosterone, and 300 μl buffer at 5°C for 18 h. Phase separation was accomplished by addition of 100 μl of a 1:80 suspension (w/v) of dried *Staphylococcus aureus* (IgGSORB; The Enzyme Center, Malden, MA), incubation for 15 min, addition of 3 ml PBS, centrifugation at 1000 × g for 20 min, decanting, and counting of radioactivity in a gamma spectrometer. Results were analyzed using an iterative least-squares weighted regression of percentage binding as described by Rodbard (1974) and expressed as ng testosterone/ml serum. Extraction efficiency ranged from 81% to 99% and averaged 91 ± 2%. The coefficient of variation within assays was 10 ± 1%; interassay coefficients of variation for 3 quality control samples containing a mean of 0.43, 2.57, and 4.81 ng/ml of testosterone evaluated in 10 assays were 22.34%, 8.84%, and 7.87%, respectively. Values obtained for water blanks and serum from castrated animals in each assay were <0.05 ng/ml. All samples from each individual animal were included within the same assay.

Testis Location and Measurement

At the time of each blood collection and following anesthesia, the testes were located and recorded as being either abdominal or scrotal, and then one testis

was measured. Length and width of the scrotal testes were measured with a millimeter ruler and by the same person throughout the study. If a testis could not be gently pushed through the inguinal canal into the scrotum, the measurements were estimated by palpation through the abdominal wall. Testis volume was calculated using the formula for a prolate spheroid, $\frac{4}{3}\pi ab^2$, with a and b being the radii of the major and minor axes, respectively.

Newly Captured Animals

Testis size was also determined at the time of capture during the normal breeding season (March and April) in 110 adult males and 26 yearling males over a 3-yr period. Males classified as yearlings were determined to have been born the previous year based on skull shape, length, body weight, and color and width of teeth (Davis and Finnie, 1975: Young and Sims, 1979). Testis size was measured again in June in 23 adults and 19 yearlings that had been housed outside for 2–3 mo following capture. Serum testosterone levels were determined in 38 of the adults at the time of capture and in 8 of them again in June of the same year that animals maintained indoors were studied.

Means are reported as mean ± SEM. Differences between groups were determined by Student's t-tests.

RESULTS

Hibernation

None of the males was observed hibernating during the 9 wk of food and supplemental light deprivation, except for a single yearling hibernating in the 8th and 9th weeks.

Testis Location

The incidence of scrotal (vs. abdominal) testes at the initiation of each examination of adult and yearling males housed inside is shown in Fig. 1. Among the 5 adults, the number with scrotal testes in December, January, March,

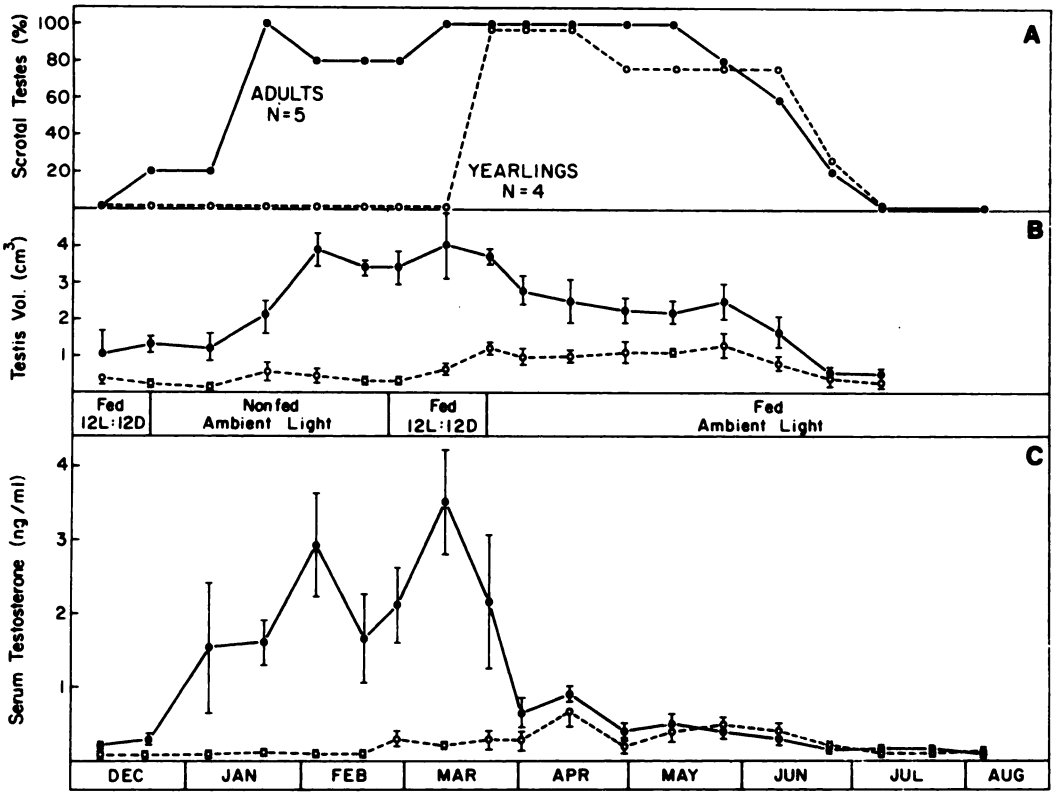

FIG. 1. Reproductive parameters in adult (•) and yearling (○) male woodchucks following maintenance outdoors for 3–5 mo and during maintenance indoors from early December through August at 42°30′N, 76°30′W. *A*. Percent of animals in each group in which testes were scrotal (vs. abdominal) following anesthesia and prior to palpation. *B*. Mean ± SEM testis volume calculated from minimum and maximum testis diameter measured in scrotum or estimated by abdominal palpation. *C*. Mean ± SEM serum testosterone concentration.

May, and July was 1, 3, 5, 5, and 0, respectively. While the majority of adults had scrotal testes in January, none of the yearlings had scrotal testes until March. Among the 4 yearlings, the number with scrotal testes in March, May, and July were 2, 3, and 0, respectively. Abdominal testes could not be palpated in 1 adult and 1 yearling in December; in July and August abdominal testes could not be moved into the scrotum in these two animals and were measured by abdominal palpation. In all other instances, abdominal testes could be moved into the scrotum for measurement.

Testis Size

Mean testis volumes of adults and yearlings maintained inside from December to August are shown in Fig. 1. In adults, mean testis volumes were 1.0–1.5 cm^3 during December and early January, increased to 2.0 cm^3 in late January, were $\geq$3.0 cm^3 during February and March, and declined from 2.25 cm^3 to 0.5 cm^3 between April and July. In individual adults, the maximum testis volumes were 3.5–5.6 cm^3 (4.8 ± 0.4 cm^3) and were recorded in early or late March, following a previous submaximum peak in volume in February (4.4 ± 0.3 cm^3) and an intervening decline in late February or early March (2.9 ± 0.3 cm^3).

Testes of yearlings were smaller than those of adults from December through early May (Fig. 1). With few exceptions, yearling testis volumes were small (<0.5 cm^3) in December through February, increased slightly (0.5–2.0 cm^3) in late March, remained so until declining in June, and were consistently small (<0.5 cm^3) in July and August.

Mean testis volumes in adults and yearlings when captured in March or April following emergence from hibernation, and when remea-

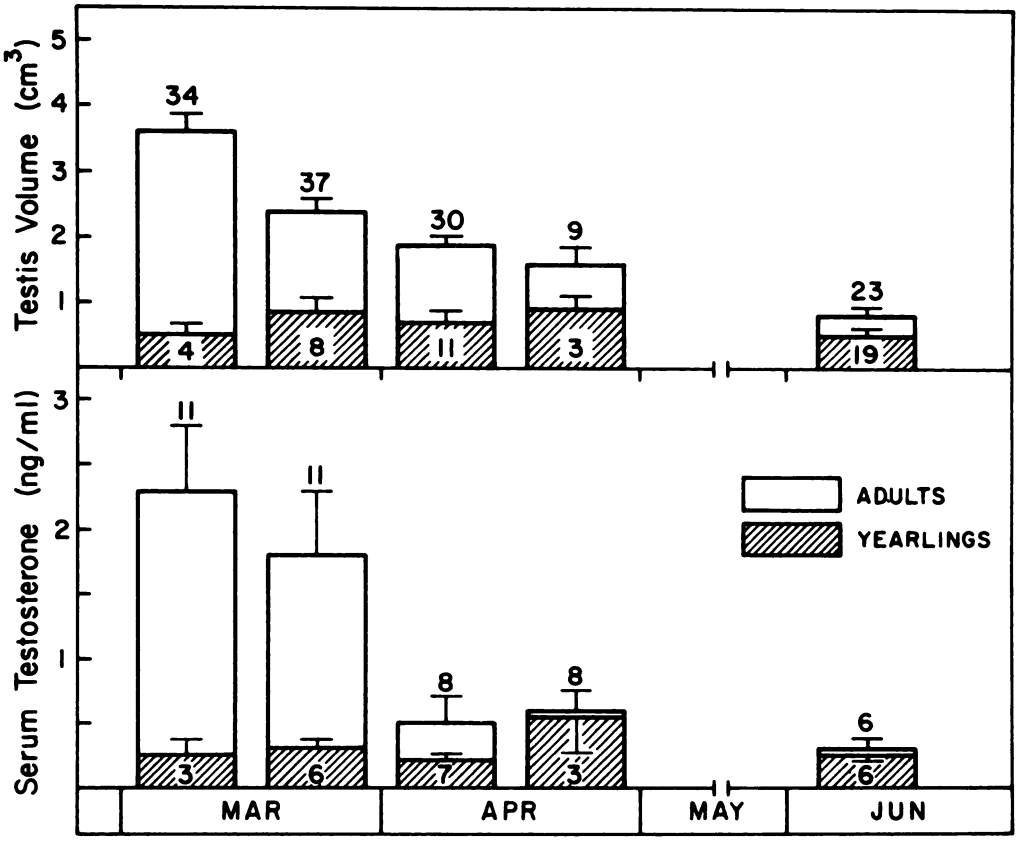

FIG. 2. Mean ± SEM testis volume at time of capture in March or April, and then in June, in adult and yearling woodchucks (*top panel*) and mean ± SEM serum testosterone concentrations in a proportion of the animals (*bottom panel*). Numbers above or within bars indicate animals per group.

sured 2–3 mo later in June, are shown in Fig. 2. For both adults and yearlings testis sizes at capture or reexamination were, in general, similar to those observed at comparable times in animals maintained inside. However, testes in adults captured in late March (2.4 ± 0.2 cm^3) were smaller ($P<0.01$) than those of adults captured in early March (3.6 ± 0.2 cm^3) (Fig. 2), whereas a comparable decline in testis size within adults maintained inside did not occur until April (Fig. 1). Mean testis sizes of newly caught adults were smaller ($P<0.05$) than those of inside-maintained adults in late March (2.4 ± 0.2 vs. 3.7 ± 0.2 cm^3), early April (1.9 ± 0.1 vs. 2.6 ± 0.3 cm^3), and again in June (0.8 ± 0.1 vs. 1.6 ± 0.4 cm^3).

Testosterone

Serum testosterone levels in adult and yearling males maintained inside from December through August are summarized in Fig. 1. Mean levels in yearlings were lower than those in adults from December through March. Testosterone in yearlings was <0.1 ng/ml from December to mid-February, and was slightly elevated but variable from late February to June. In individual yearlings, levels were >0.2 ng/ml in 3–8 of the 9 samples collected between late February and early June, with peak levels of 0.5–1.5 (0.9 ± 0.2) ng/ml occurring in April or May.

With few exceptions, testosterone levels in adults were >0.1 ng/ml and greater than those in yearlings during December, elevated but variable January through March, and then considerably lower and similar to those in yearlings in April and thereafter (Fig. 1). In individual adults, testosterone was >1.0 ng/ml in 4–7 of the 7 samples collected during January through March, with peak levels of 3.4–6.6 (5.2 ± 0.5) ng/ml occurring between early January and late March. In 4 of the 5 adults, elevations >2 ng/ml in late January/early February and again in late March were interrupted by intervening declines to <1.5 ng/ml in late February. In most instances, testosterone levels in adults were <1.0 ng/ml in April, <0.6 ng/ml in May, <0.2 ng/ml in June, and <0.1 ng/ml thereafter. Testosterone levels in 3 individual adult males are shown in Fig. 3.

Mean serum testosterone levels in wild adult males at the time of capture in March or April and again in June are shown in Fig. 2. Mean levels (ng/ml) in wild adults were slightly but not significantly lower than those in captive adults indoors in early March (2.3 ± 0.5 vs. 3.5 ± 0.7), and were similar to those in captive adults in late March (1.8 ± 0.5 vs. 2.2 ± 0.9), early April (0.5 ± 0.2 vs. 0.6 ± 0.2), and thereafter.

The variation in testosterone in 14 pairs of samples collected 1 day apart ranged from slight (0.7 vs. 0.9; 2.2 vs. 2.6 ng/ml) to considerable (0.9 vs. 5.8; 2.8 vs. 11.3 ng/ml). The mean high and low values within pairs of samples were 4.7 ± 0.9 and 1.8 ± 0.3 ng/ml, respectively. In 4 of 12 instances in which one of the paired samples had levels >1 ng/ml, the other sample did not.

DISCUSSION

In early March, there were no differences in estimated testis size between males captured shortly after emergence from hibernation in the wild and males maintained in captivity within either age group. This suggests that the completion of testicular recrudescence observed in the laboratory-maintained adults was normal in extent and timing. Whether the time course of the preceding increases in testis size and testosterone observed were similar to those occurring during hibernation in the wild could not be determined from the present study. Information on progressive changes in testicular activity during hibernation in the woodchuck have not been reported. It is possible that the reduced metabolic activity associated with hibernation might result in a slower and/or later pattern of recrudescence. However, if the length of time for spermatogenesis in woodchucks is 50 or more days, as in most other species (White, 1970; Hafez, 1970), increased testicular activity in late December and early January would be expected in males emerging from hibernation ready to breed in late February. The late December/early January onset of recrudescence in laboratory woodchucks (Fig. 1) was therefore probably similar to that occurring under natural conditions. In addition, the proven fertility of 2 of the laboratory-maintained adults indicates that the initial changes in testis volume and testosterone observed in these nonhibernating males were associated with the subsequent development of complete reproductive capability.

The results demonstrate that full testicular recrudescence can occur in the absence of hibernation. Furthermore, testis enlargement

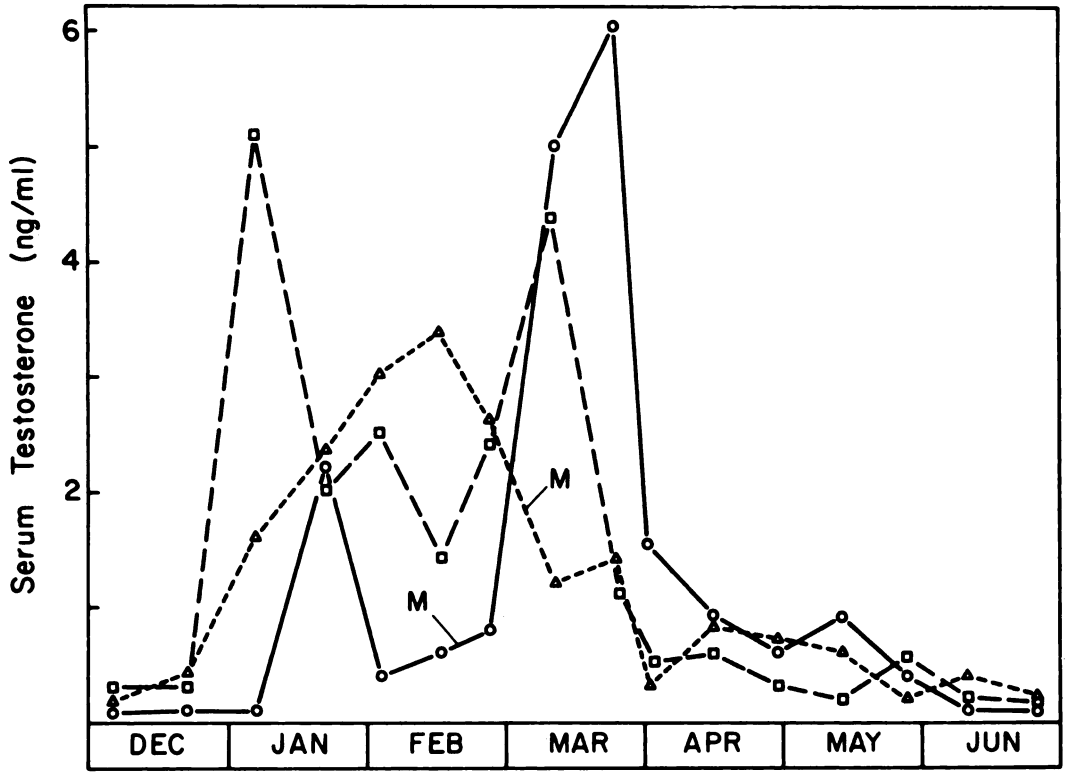

FIG. 3. Serum testosterone concentrations in 3 individual adult male woodchucks maintained indoors (as described in Fig. 1), 2 of which sired litters at matings (*M*) timed on the basis of times of parturition of their female cage mates.

and testosterone secretion, to extents greater than subsequently observed in July, occurred prior to the winter solstice and independently of two factors associated with natural hibernation, the deprivation of food and of light. The absence of observations on wild animals prior to March or on animals maintained indoors under constant conditions precludes conclusions about the relation between the withdrawal of food and supplemental light in late December and the subsequent further increase in mean testis size and testosterone levels seen in adults. However, in more recent studies comparable increases in testis size and testosterone have been observed in adults from which food was not withdrawn (Baldwin and Concannon, unpublished observations).

The absence of hibernation in the captive males was in contrast to a high incidence of hibernation in females in the same colony (Concannon et al., 1984). Elevated testosterone has been reported to terminate hibernation, prevent hibernation, and increase activity in hamsters (Hall and Goldman, 1980). Testosterone would not appear to be the cause of hibernation failure in the present study since levels were low in the yearlings, which also failed to hibernate. Nevertheless, testosterone might play a role in the emergence of adult males from hibernation in the wild since they emerge earlier than yearlings or females (Hamilton, 1934; Snyder and Christian, 1960). The differences between the sexes in the incidence of hibernation might be related to hormones other than testosterone. Most studies on hibernation in woodchucks have utilized constant-temperature rooms and food withdrawal earlier and for longer periods of time than in the present study (Davis, 1967). In limited studies using conditions similar to those of the present study, hibernation has been observed in the presence as well as the absence of food. Possibly the fluctuating ambient temperatures in the present study were less

conducive to hibernation than a constant temperature would have been.

The observation of smaller testes in males captured after mid-March than in those captured in early March agrees with the progressive decrease in testes weights previously reported for males captured in February, March, and April (Christian et al., 1972). If testis size and testosterone secretion affect the time of emergence from hibernation, capture date could be dependent on testis development. Nevertheless, the decreases in testis size and spermatogenic activity observed before and during the breeding season (Christian et al., 1972) are assumed to be representative of changes occurring within individual animals. Since there was no protracted and obvious decline in testis size in the laboratory-maintained adults until April, it is possible that tubule degeneration was slightly delayed in these animals. However, although the number of animals was limited, the testis sizes observed were, at each time studied, within the range observed in newly captured males. We therefore assume that the observed changes represent those that might be seen serially within adult males in the wild.

Prior to the end of March, both recently captured and laboratory-maintained yearlings had smaller testes and less serum testosterone than did adults. These findings, and the later timing and limited extent of subsequent increases in testis size and testosterone levels in yearling males, suggest that young males are not a significant component of the breeding population. The woodchuck therefore differs from another sciurid rodent, the golden-mantled ground squirrel, in which testosterone levels are similar in yearlings and adults (Licht et al., 1982). Christian et al. (1972) described yearling woodchucks that had enlarged spermatogenic testes during the normal breeding season, but such animals were not represented in the present study.

The levels of testosterone observed during testicular recrudescence and the breeding season in captive woodchucks were similar to those reported for other rodents, including rats (Moger, 1977), hamsters maintained on long days (Tamarkin et al., 1976; Bex et al., 1978), and two species of non-marmotine sciurids, grey squirrels (Siwela and Tam, 1981) and golden-mantled ground squirrels (Licht et al., 1982). The breeding season in woodchucks is shorter than that of both the grey squirrel (Siwela and Tam, 1981) and the golden-mantled ground squirrel (Licht et al., 1982), and the elevations in serum testosterone are correspondingly briefer in woodchucks than in either of the other two sciurid species. The start of the breeding season is earlier for grey squirrels (Siwela and Tam, 1981) and later for ground squirrels (Licht et al., 1982) than it is for woodchucks, and initial elevations in testosterone are correspondingly advanced in grey squirrels and delayed in ground squirrels when compared to those in woodchucks. In all 3 of these species, testosterone levels are elevated 1–2 mo prior to the breeding season in association with the onset of spermatogenesis, and reach peak levels shortly before or at the onset of the breeding season.

The changes in mean testosterone levels observed are most likely indicative of Leydig cell steroidogenic activity during the course of testicular recrudescence, the breeding season, and subsequent testis regression. However, testosterone profiles in individual animals obtained with the present sampling frequency must be interpreted with caution. The considerable variation in testosterone levels in samples collected one day apart suggests that secretion is episodic and that individual values may not be indicative of the extent or capacity of secretion.

The rapid decline in mean testosterone levels in the laboratory-maintained adults in late March and early April occurred about 1 mo following reexposure to supplemental lighting and reintroduction of food, and was coincident with a similar decline in levels seen in recently captured adults. The decline may serve to effect a termination of the breeding season, although mating behavior in woodchucks has not been studied. The decline occurred at the time reported for the nearly complete loss of tubular spermatozoa in woodchucks captured in the wild, and may contribute to such loss, but degenerative changes in the spermatogenic epithelium are evident early in the breeding season (Christian et al., 1972). The decline in testosterone also occurred at the same time that ovaries of similarly maintained nonfertile females underwent spontaneous luteinization (Concannon et al., 1984). Perhaps an environmental signal such as longer periods of daylight plays a role in testicular regression and decreased testosterone secretion. Long days are usually stimulatory in hamsters (Goldman, 1980) but have an inhibitory effect on testicular activity in at least one sciurid rodent, the

western chipmunk (*Eutamias sp.*) (Kenagy, 1981). Melatonin rhythms are modified by photoperiod in at least one marmotine rodent (*Marmota flaviventris*) (Florant and Tamarkin, 1984).

The small but obvious increase in serum testosterone between February and June in yearlings was such that, during April and May, levels were not different from the reduced levels seen then in adults. These findings as well as the associated increases in testis size are in general agreement with previously reported histologic observations. While the majority of yearlings have small infantile testes, from March to May they show spermatogenic activity to varying degrees and have modest to abundant numbers of small or large Leydig cells (Christian et al., 1972).

The slight increase in testis size in May was not accompanied by any detected rise in testosterone levels in either age group. While this increase in testis size can be considerable and is apparently related to an increase in interstitial tissue (Rasmussen, 1918; Christian et al., 1972), it seems that any interstitial tissue hyperplasia or hypertrophy late in the breeding season is not accompanied by increased testosterone secretion. That would be consistent with the presence of nearly nondetectable levels of testosterone in the laboratory-maintained yearlings in July and August at the same time that Leydig cells in wild yearlings were abundant and large (Christian et al., 1972).

The extent to which temperature and/or lighting conditions had effects was not examined. The testicular recrudescence in adults maintained indoors may have occurred independent of any environmental signals and the same could be true for changes occurring simultaneously in wild male woodchucks, as suggested for another species of sciurid rodent. The annual reproductive changes in male golden-mantled ground squirrels persisted for 2 or more yr under constant conditions (Kenagy, 1980; Licht et al., 1982). Similar controlled studies of the potential endogenous aspects of the annual reproductive events of the woodchuck or other species of marmot have not been reported. Environmental conditions most likely modulate any endogenous circannual rhythm, since the annual weight cycle of woodchucks was reversed in woodchucks translocated to the southern hemisphere, but the adaptation required 2 yr (Davis and Finnie, 1975).

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