

## Annual Cycles of Gonadotropins and Androgens in the Hibernating Golden-Mantled Ground Squirrel

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Ground squirrels, captured in the field, were housed at ambient temperatures of 23° (photoperiod = 10L:14D) for 13 months. Plasma was sampled at 3 to 4-week intervals and measured for gonadotropin and androgen levels. Testis size was examined monthly by laparotomy. Male ground squirrels showed clear circannual cycles in body mass, testis size, and levels of follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), and dihydrotestosterone (DHT). During summer and fall, FSH, LH and androgen levels were low, testes were undeveloped, and body mass was increasing. Testes began to rapidly enlarge in January and reached maximum size in February. A rise in FSH preceded gonadal growth but LH remained low until near the time of testis mass peak. LH remained elevated during spring while FSH levels fell and testes regressed. Plasma T and DHT levels generally paralleled LH concentrations; DHT levels were approximately one-fifth those of T levels. During winter animals lost weight but were only occasionally found in a slightly hypothermic condition. Females had elevated plasma LH levels (>1 ng/ml) predominantly in the spring but displayed no cycle in plasma FSH levels. A second group of males held at 4° for 8 months (photoperiod = simulated natural for 47° N) were regularly torpid during a hibernation season that lasted between November and May. Most (15/21) of these males did not show gonadal development by spring; these non-reproductive males had had restricted body mass gains the previous fall. Plasma FSH was low in both reproductive and non-reproductive males during fall and winter but increased in March while animals were still hibernating. FSH levels continued to increase in April only in reproductive males and reached maximal levels after hibernation was spontaneously terminated. LH titers were elevated in individual males in winter during torpor and were greater in reproductive than in non-reproductive males in May. Androgen levels were undetectable in torpid squirrels, elevated in animals sampled during periodic arousals, and elevated in most males within 3 weeks after terminating hibernation. © 1986 Academic Press, Inc.

In certain species of the rodent family Sciuridae, particularly the ground squirrels and their close relatives, cycles of gonadal development and regression occur spontaneously and persist under experimentally constant environmental conditions with a period of approximately 1 year (Heller and Poulson, 1970; Kenagy, 1980). This differs from most other seasonally breeding mammals investigated in which initiation and inhibition of gonadal growth or reversal of spontaneous gonadal maturation

requires a change in daylength (Sadleir, 1969; Zucker *et al.*, 1980). In addition to endogenous annual cycles of reproductive function, many sciurids show annual cycles of hibernation. When ground squirrels enter hibernation in late summer or early fall they have immature or regressed gonads (McKeever, 1964), yet 6-9 months later breeding occurs only a short time after emergence from hibernation (Michener, 1983). This observation implies that gonadal growth and maturation occurs in winter or early spring during the hibernation season. Little is known about the interrelations between reproductive maturation, associated hormone secretion, and

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the annual cycle of hibernation (Wang, 1982). To my knowledge no measurements of gonadotropins and few of gonadal steroid hormones (Dutourné and Saubureau, 1983; Jallageas and Assenmacher, 1983) have been reported for hibernators during deep hypothermia.

Annual cycles of hibernation and reproduction have been well described in golden-mantled ground squirrels (*Spermophilus lateralis* and the similar species *S. saturatus*) which are common in the mountains of western North America. Recent investigations have assessed the annual pattern of gonadal and endocrine function and its relation to the annual cycle of body mass. Kenagy (1980) has shown that annual cycles of gonadal size persist in golden-mantled ground squirrels in unchanging ambient conditions, and Licht *et al.* (1982) have described associated endogenous cycles in plasma luteinizing hormone (LH) and testosterone content. Zucker and Licht (1983a, b) have further demonstrated that circannual cycles of LH secretion persist in females despite gonadectomy. These studies were all done in animals held in room temperature, however, and did not assess the influence of deep, seasonal hypothermia on reproductive function. I compare here the association between annual patterns of plasma LH, gonadal steroid hormones, and follicle-stimulating hormone (FSH) with cycles of testis size and body mass in ground squirrels held in non-hibernating (warm) and hibernating (cold) conditions.

#### MATERIALS AND METHODS

Golden-mantled ground squirrels (*S. saturatus*) were trapped in the Cascade Mountains of Chelan County, Washington. *S. saturatus* is similar to and possibly conspecific with the more familiar and widespread *S. lateralis* (Hall, 1981). Animals were brought to the laboratory and provided with Purina Rodent Laboratory Chow and water *ad libitum* except where noted.

At 3- to 4-week intervals animals were weighed, and, after exposure to ether, 0.8 ml blood was with-

drawn by cardiac puncture between 1300 and 1600 PST. Blood was collected within 3 min of first handling each animal. Plasma was separated and stored at  $-20^{\circ}$ .

*Experiment 1.* Nine adult males and 12 adult females captured in May 1980 were housed singly in cages for 13 months in a constant light-dark cycle of 10L:14D (lights on at 0800 PST) and temperature of  $23 \pm 2^{\circ}$ . Plasma obtained from males was analyzed for FSH, LH, testosterone, and DHT; plasma from females was analyzed for FSH and LH. In addition, on the day after blood collection each male was anesthetized with sodium pentobarbital (6–10 mg/100 g body mass) and laparotomized to measure the linear dimensions of one testis from which mass ( $M$ , in mg) of both testes was estimated using the equation:  $M = 1.068(L \times W^2) - 0.78$  (Kenagy, 1979). Five males and 10 females survived to the end of the experiment (July 1981).

*Experiment 2.* Twenty-one males of juvenile size and appearance captured in late August 1981 were housed singly in cages and exposed to a simulated natural photoperiod (clock adjusted weekly) for the latitude of capture ( $47^{\circ}$ N) and an ambient temperature that was lowered from  $20^{\circ}$  by  $2^{\circ}$  each week until  $4^{\circ}$  was reached on 1 November. During the fall 12 animals were given rations of lab chow restricted by 20% of normal as part of another experiment (Barnes, 1984); the remaining animals had unrestricted rations. All food was removed from each animal's cage when hibernation began. Each animal was examined daily for torpor, and sawdust was placed upon hibernating animals so that the occurrence of an arousal was indicated by the next day's absence of sawdust. Animals were weighed and samples of blood were collected from September 1981 to June 1982. Squirrels in deep torpor were not anesthetized prior to sampling blood; in these cases blood samples were obtained within 1 min of touching each animal. Sixty percent of torpid animals handled in this way did not arouse after being returned to the nest. Blood was sampled from torpid individuals on 156 occasions and on 9 occasions during the normothermic period between bouts of torpor. Fifty-two randomly selected samples from torpid animals were analyzed for androgen levels, all samples were measured for FSH and LH content. Samples taken from aroused animals were distributed over the entire hibernation season. Plasma samples were assayed for FSH, LH, testosterone, and DHT. Food was replaced in each animal's cage after it spontaneously ended hibernation or on 5 May, whichever occurred first. On 18 May each animal was removed from the cold, anesthetized with ether, and the dimensions of one testis were measured as described above. Reproductive condition was continually assessed over the next months by palpating for testis size and noting whether scrotal skin was pigmented. Pigmented

scrotal skin is an indication of full testicular development (McKeever, 1964).

**Radioimmunoassays: Gonadotropins.** Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were measured using radioimmunoassay (RIA) kits provided by NIAMDD. Specific antibodies used were anti-rat FSHS-10 and anti-rat LHS-5, reference preparations for standards were FSH-RP-1 and LH-RP-1, and rat FSH 1-4 and rat LH 1-7 were radioiodinated.

RIA procedures were similar to those described by Follett *et al.* (1972). Forty microliters of plasma were incubated at 5° in duplicate with 20  $\mu$ l of antiserum (diluted 1:2000 for FSH; 1:5000 for LH) for 24–48 hr. Radioiodinated LH or FSH (5000 cpm) in 20  $\mu$ l buffer was added for an additional 12–24 hr before the addition of 20  $\mu$ l anti-rabbit  $\gamma$ -globulin (1:10 in 1:40 normal rabbit serum) for a final 24 hr. Bound and free labeled hormone were separated by centrifugation. Minimum detectable levels were 25 ng/ml for FSH and 1 ng/ml

for LH; the coefficients of variation between assays for FSH and LH were 10.9 and 15.5%, respectively.

Anti-rat gonadotropin RIA kits have been validated for use with diverse rodent species (Beamer *et al.*, 1972; Bast and Greenwald, 1974). Validation procedures for use with *S. saturatus* included demonstration of parallelism between dilutions of rat standard and dilutions of squirrel plasma and pituitary homogenates (Barnes, 1983).

**Steroid hormones.** Concentrations of testosterone and DHT were measured in plasma by RIA using procedures similar to those described by Wingfield and Farner (1975); 100–300  $\mu$ l of plasma were equilibrated with approximately 2000 cpm of each <sup>3</sup>H-steroid and extracted with 10 vol of dichloromethane. Extracts were dried, resuspended in 10% ethyl acetate in isooctane, and transferred to columns of Celite:propylene glycol:ethylene glycol (6:1.5.5:1.5 w:v:v) with Celite:water (3:1, w:v) "glycol traps." DHT and testosterone were eluted with increasing concentrations of ethyl acetate (10 and 20%, respectively) in isooctane. Fractions were dried, assayed in duplicate, and individual values were corrected for the fraction recovered after chromatography. Minimum detectable levels for an average plasma volume ranged from 40 to 50 pg/ml for testosterone and DHT. The coefficients of variation measured between assays was 12.7% for both steroids.

Statistical comparisons were evaluated using ANOVA, two-tailed *t* tests, or Duncan's Multiple Range Test where appropriate.

## RESULTS

### Experiment 1: Gonadotropins and Androgens in Non-hibernating Ground Squirrels

**Males.** Gonadotropin and gonadal steroid hormones and testis and body size showed clear annual fluctuations (Fig. 1). During summer and early fall plasma levels of LH and FSH were low and testes remained undeveloped; during this time body mass increased by almost 50% and peaked in November. Although an increase in testis size began in fall, the majority of growth did not occur until January and February. Beginning approximately 1 month prior to rapid gonadal growth, plasma concentrations of FSH increased and remained elevated during mid winter ( $P < 0.02$ , Dec, Jan, or Feb vs Nov). In contrast with FSH, LH remained low

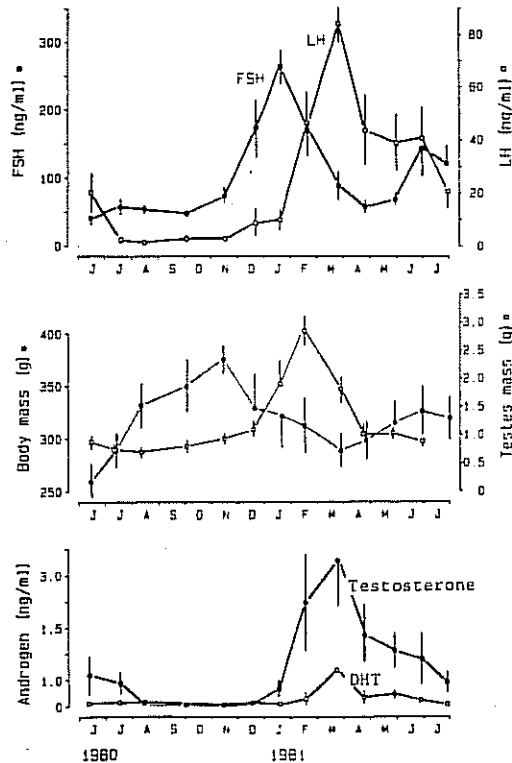


FIG. 1. Plasma levels of FSH and LH (upper panel), body and estimated testis mass (middle panel), and testosterone and dihydrotestosterone (lower panel) for male golden-mantled ground squirrels held in 10L:14D, 23  $\pm$  2 for 13 months. Values represent means  $\pm$  SE;  $n = 4$ .

during fall and winter and did not increase significantly until February ( $P < 0.05$ , Feb vs Jan) when testes were near maximally enlarged and FSH concentrations had declined. Rapid involution of testes began directly after the testis peak, and gonadal size and FSH returned to minimum levels by late April. Plasma levels of testosterone and DHT changed in parallel to LH; DHT concentration was generally less than 20% that of testosterone. Levels of LH, testosterone, and DHT remained elevated into late spring even after testes appeared to have completely regressed.

One exceptional male (excluded from Fig. 1) increased in body mass to a peak in mid July, showed maximal plasma levels of FSH in August, and underwent full gonadal enlargement by September. During the 7 months from October to March his testes decreased gradually in size and only became fully regressed (<500 mg) by April. The maximum in plasma LH concentration in this animal occurred in February.

**Females.** The seasonal course of body mass for females was similar to that of males, with a maximum in November and minimum in April. Detectable levels of FSH (>25 ng/ml), however, were found in only 6 of 128 plasma samples taken over the year, and there was no seasonal pattern to the distribution of these 6 values. Concentration of plasma was elevated (1.5–27 ng/ml) in the majority of females between February and May and mostly undetectable in other months of the year (Fig. 2). The spring increase of LH in individual females occurred before or at the seasonal nadir of body mass.

#### *Experiment 2: Gonadotropins and Androgens in Hibernating Ground Squirrels*

Ground squirrels held in an ambient temperature of 4° were regularly torpid during a hibernation season that began between October and December and lasted 4 to 7 months. This contrasts with the behavior of

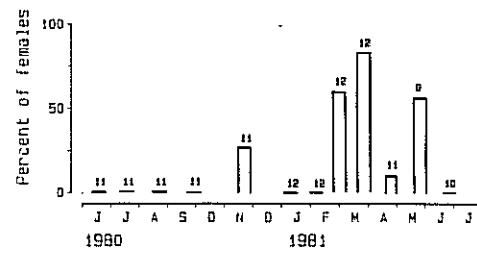


FIG. 2. Percentage of female golden-mantled ground squirrels that showed detectable levels of LH (>1 ng/ml) over 13 months while held in 10L:14D and 23 ± 2°. Numbers above bars represent sample size.

ground squirrels held in 23° (experiment 1); at bi-weekly checks these animals were found only occasionally in a slightly hypothermic state during the months when body mass was declining.

In hibernating animals in experiment 2 torpor was interrupted at intervals of several days to 2 weeks by spontaneous arousals, in a pattern similar to other hibernating ground squirrels (Twente and Twente, 1967). During each arousal body temperature was elevated from the level of deep torpor (4–8°) to the normothermic level (36–38°) and then remained normothermic from 8–24 hr before torpor was resumed. By mid-May, 15 of 21 males had not undergone gonadal development, and these males remained immature during the subsequent summer. The six other males did show reproductive maturation. In general, only males that had free access to food and that surpassed approximately 300 g in body weight before entering hibernation became reproductively mature. This relationship between reproductive maturation and body size may be due to a dependency of maturation upon the extent of endogenous energy reserves accumulated previously (Barnes, 1984). For the purpose of examining the hormonal correlates to reproductive cycles, data from males that underwent maturation were considered separately from data from males that did not.

In males that showed gonadal development ("reproductives") plasma levels of FSH declined significantly ( $P < 0.05$ , Dec

vs Nov) after the beginning of hibernation in late fall (Fig. 3, upper panel), remained low throughout winter, and increased significantly in March ( $P < 0.001$ , Mar vs Feb). In five of six males FSH levels reached a maximum (130–200 ng/ml) only after spontaneous termination of hibernation in spring. In two of these five this maximum occurred during the first week of activity (Table 1). In the sixth reproductive male FSH was not observed to increase above 74 ng/ml (Table 1, No. 102).

In males that remained sexually immature ("non-reproductives") the course of plasma FSH was similar to that of reproductive males during fall and winter (Fig. 3, lower panel): FSH levels declined as animals entered hibernation and increased significantly in February and March ( $P < 0.01$ , February vs January). In spring, however, FSH levels did not increase above 100 ng/ml and instead declined to the

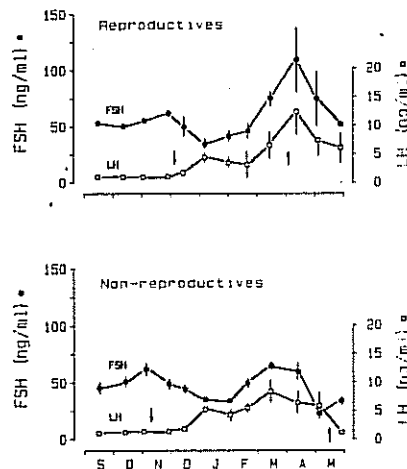


Fig. 3. Plasma levels of FSH and LH in male golden-mantled ground squirrels that underwent gonadal maturation "Reproductives,"  $n = 6$  or remained reproductively quiescent "Non-reproductives,"  $n = 15$ . Animals were kept in an ambient temperature of  $4 \pm 2^\circ$  after 1 November and in a simulated natural photoperiod (see text). Values represent means  $\pm$  SE. Downward arrows represent the date at which one-half of the animals in each group had begun hibernation; upward arrows designate when one-half of each group had terminated hibernation.

TABLE 1  
FSH AND TESTOSTERONE LEVELS IN PLASMA OF REPRODUCTIVE MALE GOLDEN-MANTLED GROUND SQUIRRELS BEFORE AND AFTER THE TERMINATION OF HIBERNATION

Squirrel no.	Day relative to last day torpid (=0)	FSH (ng/ml)	Testosterone (ng/ml)
101	-25	74	<0.05
	1	<25	<0.05
	25	130	0.242
	48	54	0.585
102	-25	39	0.051
	1	70	<0.05
	27	74	0.050
	51	34	0.412
	72	54	0.679
132	-24 <sup>a</sup>	25	2.033
	2	<25	0.369
	25	135	0.471
113	-23	78	<0.05
	3	175	0.052
	26	54	0.273
135	-19	56	<0.05
	7	160	2.089
	31	34	5.388
	54	50	20.425
	60	42	1.167
116	-13	78	<0.05
	13	200	<0.05
	37	54	8.479
60	42	1.167	

<sup>a</sup> Blood sampled during the normothermic period of an arousal.

lowest mean value of the season in early May. FSH levels in reproductive males were significantly higher than those in non-reproductive males in April and May ( $P < 0.02$ ).

Plasma LH was undetectable during fall in all males (Fig. 3). After animals entered hibernation LH concentrations in plasma became elevated in some individuals for the duration of the hibernation season; values in torpid males ranged from 1 to 30 ng/ml. Plasma LH in reproductive males was significantly higher than that in non-reproductives in late May ( $P < 0.01$ ).

Plasma levels of testosterone and DHT were essentially undetectable in animals

prior to hibernation and in torpid animals during the hibernation season (Table 2), including in those animals with elevated LH levels. In plasma sampled from normothermic animals during an arousal, mean levels of testosterone were significantly elevated ( $P < 0.001$  in comparison to levels in torpid animals) with maximal values in aroused squirrels of over 2 ng/ml (Table 2). Levels of testosterone were elevated in two of five reproductive males sampled within the first week of activity after spontaneous termination of torpor (Table 2). Testosterone in non-reproductive males was also elevated in samples taken over the first 3 weeks after hibernation (Table 2).

#### DISCUSSION

This study describes the temporal interrelations between the annual cycles of gonadotropins and androgens in plasma, gonadal growth, body mass, and hibernation in captive golden-mantled ground squirrels. In males the annual cycle consists of reproductive quiescence and body mass gain during summer and fall, followed in winter by gradual loss of body mass accompanied by increases in plasma FSH and testicular development. This culminates in a period of elevated LH and androgen levels and reproductive maturity in spring. In females, increased levels of LH were apparent

mainly between late winter and early spring, a time which encompasses the breeding season of natural populations (Kenagy and Barnes, submitted for publication). Similar annual cycles of LH in female golden-mantled ground squirrels have been previously demonstrated only in ovariectomized animals (Zucker and Licht, 1983a); attempts to demonstrate seasonal variation in plasma LH in intact female *S. lateralis* were unsuccessful (Zucker and Licht, 1983a). Female ground squirrels are thought to be induced ovulators (Moore *et al.*, 1934; Asdell, 1964) but are capable of showing spontaneous annual cycles of ovarian development and vaginal opening (Simmons, 1937; Heller and Poulson, 1970; Zucker and Licht, 1983a) of which the spring elevations of LH reported here may be one component. In individual females the first occurrence of elevated LH in spring came just before or at the same time as the end of the hibernation season was indicated by an increase in body mass. In the field females mate within only a few days after their emergence from hibernation (Bronson, 1977).

These cycles occurred in both sexes in the absence of seasonally changing environmental cues; thus these observations support and extend to FSH levels in males and LH levels in females the conclusions of

TABLE 2  
PLASMA LEVELS OF TESTOSTERONE IN MALE GOLDEN-MANTLED GROUND SQUIRRELS PRIOR TO THE BEGINNING OF HIBERNATION, DURING HIBERNATION, DURING PERIODIC SPONTANEOUS AROUSALS, AND WHILE ACTIVE BETWEEN 1 AND 60 DAYS AFTER THE END OF HIBERNATION<sup>a</sup>

	Active before hibernation	Hibernating	Aroused	Active after hibernation	
				Reproductives	Nonreproductives
Mean	0.061	0.063	0.534	8.824	0.527
SE	0.005	0.005	0.250	4.824	0.209
<i>n</i>	13	52	9	17	14

<sup>a</sup> Reproductive males underwent gonadal maturation; Non-reproductive males remained reproductively immature. Values are expressed in ng/ml; *n* refers to number of samples.

Kenagy (1980), based on testes size, and Licht *et al.* (1982), based on plasma LH and testosterone levels, that golden-mantled ground squirrels have endogenous circannual rhythms of reproductive function.

The relative timing of the increases in plasma FSH and LH during winter varied in males according to whether or not they hibernated. In adults that did not exhibit deep torpor the increase and maximum in circulating levels of FSH came 1 to 3 months before the major increase in LH. A temporal separation between the rise in plasma levels of the gonadotropins similar to that reported here for non-hibernating ground squirrels has been previously noted during spontaneously gonadal recrudescence in the golden hamster (Turek *et al.*, 1975; Matt and Stetson, 1979) and during gonadal maturation in rats (Swerdloff *et al.*, 1971; McCann *et al.*, 1972); however, the degree of separation between the rise in FSH and LH levels in the ground squirrels undergoing maturation shown here is perhaps the most pronounced of these. The yearly activation of reproductive function in golden-mantled ground squirrels thus could prove to be a useful model in exploring independent regulation of secretion of gonadotropins (Grady and Schwartz, 1981).

In males that hibernated and that became reproductive, maximum concentrations in FSH and LH appeared coincidentally and occurred only after animals had terminated torpor. Before the end of the hibernation season in March, however, FSH levels in these males did increase significantly from the mid-winter minima; average concentrations of FSH in plasma of torpid animals reached nearly 80 ng/ml. Whether this level of circulating FSH stimulated gonadal growth is unknown since successive testis measurements were not made in these animals. The physiological potency of hormones in hibernators might be altered at low body temperatures—maximum

binding of FSH to testicular receptors occurs at intermediate temperatures in amphibians that breed at intermediate ambient temperatures (Kubokawa and Ishii, 1984). However, similarly high (although not sustained) levels of FSH in hibernating non-reproductive males during March did not result in gonadal development. When testicular growth and differentiation occurs in relation to bouts of torpor and the emergence from hibernation awaits further investigation.

The later rise of FSH in animals in experiment 2 as compared to experiment 1 could also relate to the younger age of animals in experiment 2: Juvenile ground squirrels in natural populations enter and emerge from hibernation later than do adults (Bronson, 1979) and may have a generally delayed schedule of reproductive maturation.

The failure of many of the males in experiment 2 to mature is apparently related to their low levels of energy stores (fat) at the end of the hibernation season (Barnes, 1984), and the differences in the patterns of gonadotropin levels between reproductive and non-reproductive males may reflect this influence. How or when energy balance affects the development of reproductive condition in ground squirrels is unknown, but it is interesting that the course of gonadotropin secretion in non-reproductive males is similar to that in subsequently reproductive males until late in the hibernation season. It was not until March that FSH levels increased to the seasonal maximum in reproductive males whereas FSH did not increase in non-reproductive males. This suggests that preparation for reproduction begins in early spring, resulting in termination of hibernation and an increase in circulating gonadotropin levels. Animals that do not reproductively mature do not exhibit elevated FSH and LH, and they continue to hibernate.

Levels of testosterone and DHT did not

increase in the plasma of torpid animals earlier than 9 days before the last day of torpor when the sample closest to hibernation end was taken. On the other hand testosterone was elevated as soon as the second active day after hibernation in both reproductive and non-reproductive males. Elevated testosterone levels in captive male Belding ground squirrels also only occur after torpor has ended (Bushberg and Holmes, 1985). In hibernating hamster species a general inverse relationship has been observed between hibernation and reproductive activity (Jansky *et al.*, 1984), and specifically Hall and Goldman (1980) have shown that testosterone or DHT inhibits torpor in Turkish hamsters, and they suggest that increasing blood androgen may "signal" the termination of hibernation (Hall *et al.*, 1982).

The present data suggest either that the elevation of plasma gonadal steroid hormones occurs during the last week of hibernation or, alternatively, that androgens act to regulate hibernation by preventing the return to torpor after an arousal. The latter possibility is supported by the observation that, although high levels of circulating LH were present in torpid animals, elevated testosterone levels only were found in animals sampled at normothermic body temperature during an arousal or after the end of hibernation. If testicular endocrine responsiveness is vastly reduced or absent at low body temperatures, then elevated LH in torpid animals would only stimulate the testis to secrete androgens in conjunction with an arousal. A temperature dependence of the steroidogenic function of the testis has been demonstrated in ectotherms (Licht, 1972), and *in vitro* experiments reveal a similar insensitivity to stimulation of testes from torpid squirrels when tested at low temperatures (Barnes, 1984a).

The elevated levels of testosterone found in ground squirrels during periodic arousals

in the present study were not associated with the cessation of hibernation. Blood was only sampled once during arousal, however, and the elevated levels measured may be a transitory response of the gonads to circulating LH accumulated during a torpor bout. What distinguishes periodic from terminal arousals in hibernating ground squirrels and hamsters may be the sustained elevation of plasma testosterone levels in response to sustained LH secretion by the pituitary.

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