Effects of Arousal from Hibernation and Plasma Androgen Levels on Mating Behavior in the Male Big Brown Bat, *Eptesicus fuscus*

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ABSTRACT

The effects of arousal from hibernation and presence of plasma androgen on the expression of mating behavior in male big brown bats (*Eptesicus fuscus*) were tested in a captive population exposed to seminatural conditions in central Alabama. In the mild winter of 1994–1995, flight cage temperatures never fell below 10°C. Bats were never observed to enter sustained (over 2 d) torpor. They were also never observed to mate. Unmanipulated, sham-operated, and gonadectomized males and unmanipulated females were exposed to 6 d of 4°C. All individuals appeared torpid, and body temperatures of monitored bats fell at least 15°–20°C. Plasma androgen levels of torpid unmanipulated, sham-operated, and gonadectomized males averaged 25.4 ± 9.2, 19.7 ± 9.1, and 1.5 ± 0.25 ng/mL, respectively, and did not differ significantly from levels for the same groups 1 mo previous to induced torpor. When animals were returned to 23°C, 57% of unmanipulated, 40% of the sham-operated, and 33% of gonadectomized males displayed mating behavior upon arousal. Almost all matings occurred within 48 h of arousal, the majority in the first 3 h. Males not exposed to low temperatures were not observed to mate. Although individuals from all three treatments mated, gonadectomized males averaged fewer mounts and copulations per individual. Androgen levels declined significantly from torpor levels in all groups 48 h after arousal. Thus, an extended period of low body temperature and arousal appeared to be a short-term activator of sexual behavior in the big brown bat; unmanipulated males were more strongly affected by this stimulus than gonadectomized males.

Introduction

Reproduction in many investigated species is characterized by the close temporal association of three factors: gonadal recrudescence, sex steroid production, and reproductive behavior. Indeed, sexual behavior in many species is dependent on the presence of sex steroids; increased levels of sex steroids increase the probability of sexual behavior being expressed in the presence of the appropriate stimuli (Crichton et al. 1991). However, a large number and variety of animals, ranging from musk shrews and bats to turtles and snakes, exhibit an asynchronous or dissociated pattern of reproduction; sexual behavior occurs when gonads are regressed and circulating sex steroid levels are basal (Crews 1984). The best-studied organism displaying this pattern is the male red-sided garter snake, *Thamnophis sirtalis parietalis*. The expression of courtship behavior in male garter snakes has been documented to be relatively independent of the short-term, activational effects of testosterone (Crews et al. 1984; Crews 1991). Instead, it appears that prolonged exposure to low temperatures followed by subsequent warming is the critical stimulus in eliciting courtship behavior in garter snake males when they are presented with attractive females (Khrohmer and Crews 1989).

Many species of vespertilionid and rhinolophid bats exhibit an asynchronous or dissociated pattern of reproduction, but there have been almost no studies on the factors stimulating mating behavior in these animals (Racey 1982; Crews 1984; Mendonça et al. 1996). Sexual activity in these species occurs in the autumn as well as throughout the winter during periodic arousals from hibernation (Wimsatt 1945; Thomas et al. 1979; Racey 1982; Wai-ping and Fenton 1988; Gardner and Mendonça 1997). At the time of mating, these males have regressed gonads (Gustafson 1979).

Seasonal changes in androgen levels have been described for some of these species. During the winter mating period, androgen titers are basal for the seasonal cycle; however, these basal levels are relatively high, ranging from 2.5 to 83 ng/mL (Racey 1974; Gustafson 1979; Gardner and Mendonça 1997). This range is two to five times higher than the basal level range found in other mammalian species (Gustafson and Shemesh 1976; Setchell et al. 1994). It has been suggested that these high basal levels are sufficient to activate and maintain sexual behavior, as well as secondary sexual organs and glands, in males throughout the winter (Racey 1974). Mendonça et al. (1996) housed big brown bats (*Eptesicus fuscus*) in a large flight cage exposed to natural ambient conditions. They found that males gonadectomized 3 mo before the onset of mating activity (but after the androgen peak) copulate in the same proportion as did unmanipulated males upon periodic arousal from hibernation. This result suggests that activation of sexual behavior

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in male big brown bats may not be mediated by gonadal sex steroids alone, paralleling the results found for the male red-sided garter snake. Anecdotal observations of a population of big brown bats in central Alabama indicate that mating activity occurs when ambient temperatures increase and bats arouse from an extended period of hibernation (Mendonça et al. 1996; Gardner and Mendonça 1997). This apparent stimulation and synchronization of mating behavior by temperature is reminiscent of the activation of sexual behavior in male red-sided garter snakes (Crews 1984). However, Mendonça et al. (1996) did not directly test the effect of temperature change on the expression of mating behavior, nor did they obtain blood samples of males during hibernation and after arousal to determine changes in plasma sex steroids.

The present study explores in greater detail the relationship between arousal from a hibernation state, circulating androgen levels, and the expression of mating behavior in the male big brown bat, *Eptesicus fuscus.*

Material and Methods

Animals

Male and female big brown bats were collected in Butler County, Alabama, in October 1994. After transport to the Auburn University campus, the bats were sexed, weighed, and individually tagged with wing bands. Average weights of males and females were 17.6 ± 0.7 g ($N = 33$) and 20.2 ± 0.2 g ($N = 51$), respectively. Reflective number tags were glued to their backs with surgical skin bond to aid in identification during behavioral observations. Bats were housed in a 3 × 3 × 2 m flight cage constructed of screen and wood. This cage was situated in a cement block building and exposed to natural photoperiod and temperatures that were similar to, but averaged slightly higher than, ambient. Mealworms and water, each supplemented with vitamins, were provided ad lib. daily.

Surgery

A subset of the captured animals were anesthetized with metafane and underwent gonadectomy ($N = 6$) or a sham operation ($N = 5$) in early November 1994 (males had already undergone peak androgenesis; Gardner and Mendonça 1997). In both groups, a small incision was made in each of the scrotal sacs. Males were gonadectomized by removing testes from the sacs and ligating and cutting the spermatic cord. In sham-operated animals, the testes were manipulated and replaced within the sac rather than detached as in the gonadectomized males. Scrotal sac incisions were then sutured, and all animals received Ancep, a broad-spectrum antibiotic.

Temperature Manipulations

During the course of our experiment, flight cage temperatures, monitored with a Tempscribe 7-d recorder (Bacharach, Pittsburgh, Penn.), averaged 14°C, with a high of 19°C and a low of 10°C. On February 5, 1995, a subset of bats (unmanipulated males: $N = 7$; sham-operated males: $N = 5$; gonadectomized males: $N = 6$; and unmanipulated females: $N = 10$) were removed from the flight cage and placed in a 110-L aquarium, which was then placed in a 4°C walk-in cold room. The aquarium had a fine wire mesh screen taped to the back wall to enable animals to roost and mate in view of a video camera. The cold room had the same light/dark cycle (12L : 12D) as bats were experiencing in the flight cage.

Three of the males (one unmanipulated and two gonadectomized) each had a dime-sized patch of the fur on their backs shaved and a temperature transmitter (BD-2T transmitters from a Holohil Systems, Carp, Ontario) affixed to this area with surgical skin adhesive. Transmitters were calibrated in water baths between 5° and 43°C before attachment. This procedure provided accurate temperature readings to 17°C, below which readings lost their resolution.

After placement in the 4°C walk-in cold room, the aquarium was undisturbed for 6 d. Body temperatures were monitored throughout the experiment from an adjacent room with a Wildlife Materials (Carbondale, Ill.) TR2000S telemetry system. On day 6 of the experiment, bats were bled in the cold room. This disturbance initiated arousal of the bats, as indicated by a rise in body temperature. After bleeding, the males were quickly returned to the aquarium, and it was immediately transported to the flight cage. A second blood sample was collected 48 h after arousal.

The other bats in the captive colony (15 males and 41 females) remained in the flight cage for the duration of the experiment and were not exposed to a prolonged episode of lowered temperatures (and thus did not experience torpor). Daily behavioral observations of the flight cage individuals were made concurrently with those in the aquarium that had experienced torpor.

Blood Collection

Blood was collected by cardiac puncture with a heparinized 1-mL syringe and a 26-gauge needle. Approximately 200–300 μL of blood was obtained at each bleeding. Males were bled 4 wk before exposure to 4°C (pretorpor), after 6 d of exposure to 4°C (torpor), and 48 h after removal from 4°C (postarousal).

Behavioral Observations

After removal from the walk-in cold room and placement within the flight cage, the aquarium was oriented in front of two low-light video cameras (a Sony SSC-S20 and a Pelco

PT506-24DT) linked to a videocassette recorder. All bat activity was continuously recorded for 96 h.

Videotapes of the aroused bats were carefully reviewed, and each individual's behavior was assessed. Bats were identified by their reflective number tags, and mating behavior for males was categorized as either an attempt or a copulation. An attempt was any approach by a male to a female that immediately resulted in a mount (purposeful crawling onto a female's back and remaining completely on the female's back by grasping and/or biting her neck, back, or shoulders); such a mount was included in the attempt category independent of subsequent success. A successful mount was scored as a copulation (the male remained on the female, grasping her neck and shoulders, and exhibited stereotypical pelvic thrusting movement associated with intromission).

Steps undertaken to observe mating behavior of flight cage bats included the following: daily 1–2 h observation periods at dusk, which had in previous years been a time of high mating activity in our captive colony, and 10 all-night observation periods during conditions that previously had been associated with high mating activity (increases in ambient temperatures after a 2–3-d period of low temperature).

Radioimmunoassay

Blood was centrifuged, and the separated plasma was removed and frozen at 20°C for later hormone analysis. Extraction and radioimmunoassay followed the protocols detailed in Mendonça et al. (1996). Plasma was incubated with 640–800 Bq of the steroid to be measured for 1 h to assess extraction efficiency. Plasma was then extracted with diethyl ether, dried down under nitrogen gas, and resuspended in phosphate buffer. The samples from males were aliquoted at two different dilutions and incubated overnight at 4°C with tritiated testosterone and a testosterone antibody (T3003, Wein Laboratories, Succassana, N.J.) that cross-reacts 49% with dihydrotestosterone. Thus, steroid assay results are presented as total androgens measured. However, other studies have indicated that dihydrotestosterone levels are low in the bats that have been examined (Gustafson and Damassa 1985), and that the total androgen titer is a relatively accurate assessment of testosterone levels. A third aliquot was used to determine percent extraction efficiency. Values were corrected for volume of plasma and efficiency of extraction and expressed in nanograms per milliliter of plasma. Percent extraction efficiency averaged 92%. Intraassay variation averaged 6.2%. Sensitivity of the assay was 10 pg/mL.

Statistics

The percent of individuals that were observed to copulate within a treatment group was calculated and frequencies among treatment groups were compared by chi-square test (Siegel and Castellan 1988). The hormone levels were tested for heterogeneity of variances. If variances were heterogeneous, values were log transformed and then analyzed by a repeated ANOVA. The relationship between plasma androgen levels of aroused bats and number of mounting attempts or copulations was analyzed by regression analysis (Sokal and Rohlf 1981).

Results

Torpor

The transmitter-equipped bats (two gonadectomized and one unmanipulated) all maintained elevated body temperatures (approximately 33°C) for approximately the first hour of exposure to 4°C. Then, in the following 2 h, body temperatures fell 5°–10°C in all three individuals, with the unmanipulated bat exhibiting the greatest decrease (a 10°C vs. a 6°C difference; Fig. 1). After 4 h of exposure, body temperatures of the two gonadectomized bats rose to initial levels for a short while (approximately 2 h) and then fell 15°–20°C below initial body temperatures (Fig. 1). The unmanipulated individual had reached an asymptote reading 11 h earlier. Temperatures remained stable at these lower temperatures for 114 h (until males were bled and removed from 4°C). These asymptote levels were the lower resolution limits of each of the transmitters. Body temperatures may, in fact, have been lower than the transmitters were indicating for at least two of the bats (the unmanipulated and one of the gonadectomized males).

Mating Behavior

Unmanipulated, sham-operated, and gonadectomized males did not significantly differ from one another in the proportion of males mating at least once (Fig. 2; $\chi^2 = 0.8$, df = 2, $P = 0.67$). When compared with gonadectomized males, unmanipulated and sham-operated males that did mate exhibited a higher mean number of attempted mounts ($\bar{X} \pm$ SD: 6.3 ± 2.8, 5.0 ± 4.0, and 2.5 ± 0.5, for unmanipulated, sham-operated, and gonadectomized males, respectively) and copulations (3.8 ± 2.1, 4.0 ± 3.0, and 2.0 ± 0.0). However, because of the small sample and high variance, these differences were not significant. Unmanipulated, sham-operated, and gonadectomized males mounted and copulated with multiple females, and individuals of the three groups mounted other males.

Mating attempts varied in relation to time from arousal. The first mount occurred within 90 min of removal of the bats from the walk-in cold room. Of the 45 total mounts observed, 68.9% occurred within 24 h of arousal (Fig. 3), with the majority of these (61.2%) occurring within the first 3 h. All of the mating attempts by gonadectomized males occurred in this first 24-h period. The majority of the remaining attempts (26.6%) occurred within the 24–48-h interval after arousal.

During this time period, none of the bats that had remained

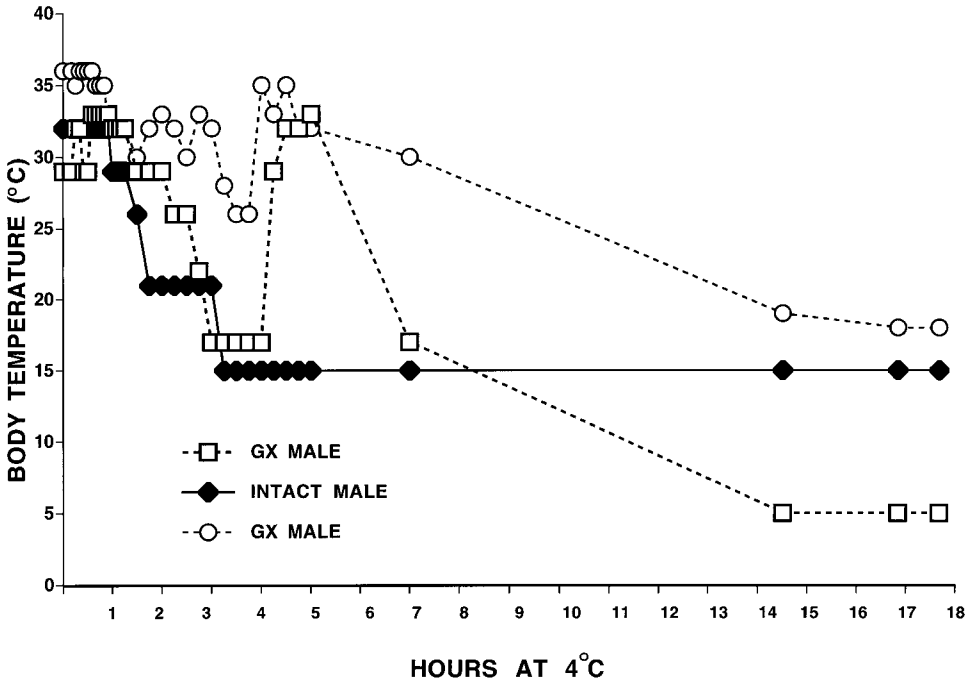

Figure 1. Body temperature profiles for three male big brown bats (one unmanipulated [*INTACT*] and two gonadectomized [*GX*]) in the first 18 h of exposure to 4°C.

in the flight cage (i.e., not exposed to a 6-d interval of 4°C) were observed to mate.

Androgen Titers

Unmanipulated and sham-operated males exhibited significantly higher androgen levels than gonadectomized males 4 wk before the experiment, while in torpor, and 48 h after arousal (Fig. 4; $F = 2.8$, df = 2, 12, $P = 0.02$). Androgen levels declined significantly between torpor and arousal ($F = 9.38$, df = 1, 14, $P = 0.01$). There was no correlation between androgen level and number of attempted mounts ($r = 0.18$, $P = 0.5$) or number of copulations ($r = 0.18$, $P = 0.5$).

Discussion

Timing of Mating

Bats from northern latitudes generally mate in autumn and upon periodic arousals from hibernation. Sperm has been found in female tracts and/or copulation has been observed as early as August–September in some species (e.g., *Pipistrellus pipistrellus* [Racey and Tam 1974]; *Myotis lucifugus* [Thomas et al. 1979; Waiping and Fenton 1988]). In California, peak copulatory activity in western big eared bats (*Plecotus townsendi;* formerly *Corynorhinus rafinesquei*) occurs in November–February (Pearson et al. 1952). In Alabama, recently inseminated females are present in wild populations of big brown bats as early as November and are still found in March (M. Mendonça and J. Kolb, unpublished observations). Mating is not observed in our captive population, however, until December and is most pronounced in January and February upon natural arousal from hibernation (Mendonça et al. 1996). Similar timing of mating occurs in the wild population of big brown bats inhabiting the attic where the captive population is housed (Mendonça et al. 1996; M. Mendonça and J. Kolb, unpublished observations).

Other bat species have also been observed to mate upon arousal. Mating activity has been noted within 1–2 h of disturbance of natural populations of hibernating little brown bats, *Myotis lucifugus.* However, these copulations are not thought to be common (Wimsatt 1945; Thomas et al. 1979). Yet reports of winter mating are widespread (Wimsatt 1945; Pearson et al. 1952; Thomas et al. 1979; Racey 1982). When animals have been artificially hibernated and then placed in warmer temperatures, a few studies have reported subsequent mating activity (e.g., Pearson et al. 1952).

Effect of Temperature Manipulation

McNab (1974) indicated that big brown bats need a microclimate of temperature of 7°C to hibernate. Thus, it was not surprising that big brown bats held in the flight cage were observed neither to enter extended torpor bouts nor to mate despite repeated, all-night observation sessions. Since observation was not continuous, it is possible that some sporadic mating activity went unobserved. However, high mating activ-

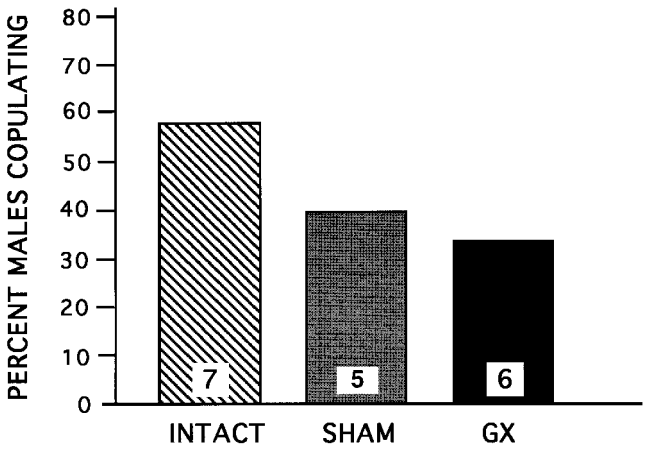

Figure 2. Percent of males from the three treatment groups (unmanipulated [*INTACT*], sham-operated [*SHAM*], and gonadectomized [*GX*]) that exhibited mating at least once in the 96-h test period. Numbers within the bars are sample sizes.

ity during the same time frame was observed in previous, more seasonal years.

To test whether exposure to a period of low temperature and subsequent arousal could stimulate mating activity, the males, both gonadectomized and intact, were subjected in this study to artificially low temperatures (4°C for 6 d) in early February. They readily entered a hibernation state. When males and females reached normal body temperatures within 1 h of arousal, mating activity by a proportion of the bats was soon observed. Almost all of the mounts and copulations occurred within 48 h of arousal. Eighty-five hours after arousal, mating ceased in the aquarium. During the period of increased mating activity for the artificially hibernated bats, none of the flight cage bats, which had experienced neither deep torpor nor low ambient temperatures, were observed to mate. However, these bats were not caged in an aquarium as were the bats exposed to low temperatures. It may be that prolonged captivity (6 d) in a relatively small area (e.g., the aquarium) increases the probability of mating. We do not think this is a tenable explanation, however, since we have previously housed animals in this way at this time of year and have not observed an increase in mating over that seen in the flight cage itself (M. Mendonça, unpublished observations). Another way in which flight cage bats differed from aquaria bats was that they were not exposed to females that had undergone torpor. It may be that factors associated with torpor and arousal can enhance the attractiveness of females.

Exposure to a period of temperature change relatively late in the winter appeared to stimulate mating behavior in males of both unmanipulated and gonadectomized groups (either directly or indirectly, by increasing female attractiveness). However, the response (in terms of number of mounting attempts and copulations) was more pronounced, though not significantly so, in the unmanipulated group.

Effect of Androgen

Elevated levels of testosterone have been demonstrated to inhibit entrance into or to terminate hibernation in other hibernating mammals (Lee et al. 1990; Ruby et al. 1993). However, unmanipulated and sham-operated big brown bats exhibited relatively elevated androgen levels (i.e., $\bar{X} = 25.4 \pm 9.2$ and 19.7 ± 9.1 ng/mL, respectively) while in torpor. These levels were equivalent to those exhibited before torpor and significantly higher than those 48 h after arousal. However, it has yet to be established whether the levels observed in our captive males are a true indication of plasma androgen titers during hibernation in bats or if they are an artifact of exposure of these males to higher ambient temperatures before sudden placement at 4°C. Still, it is clear that despite these relatively high (compared with other mammals) basal levels of androgen, intact males entered and maintained a torpid state just as readily as the gonadectomized males (whose mean androgen levels was < 1 ng/mL).

We measured total androgen, which included both unbound (free) testosterone and testosterone bound to specific sex-steroid-binding protein. The free amount of the sex steroid is thought to be the physiologically relevant measurement of effectiveness. Thus, our total androgen measures may be overestimating the amount of testosterone available to induce behavioral changes. Yet, a series of studies have documented that levels of sex-steroid-binding protein are low during the winter months, even in samples from big brown bat males in northern portions of its range (Damassa and Gustafson 1984; Gustafson and Damassa 1987). If the same is true in our captive, southern population of big brown bats, then there is, in fact, a great deal of free testosterone available, again indicating a general insensitivity to androgen in relation to expression of hibernation. These relatively elevated androgen levels are not essential to maintenance of sexual behavior during the winter months. There were gonadectomized males that exhibited a complete

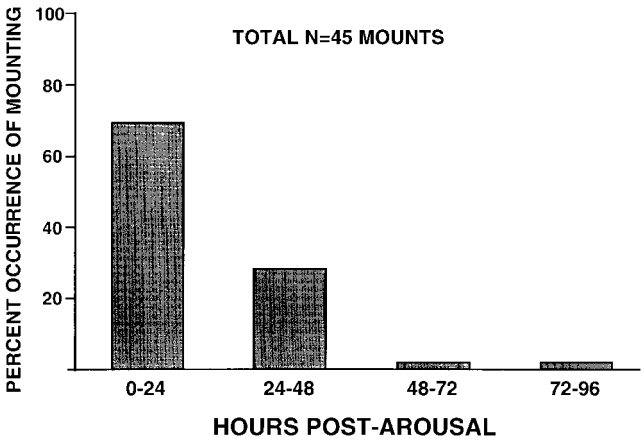

Figure 3. Distribution of the total attempted mounts by males in the 4 d after removal of male big brown bats from 4°C.

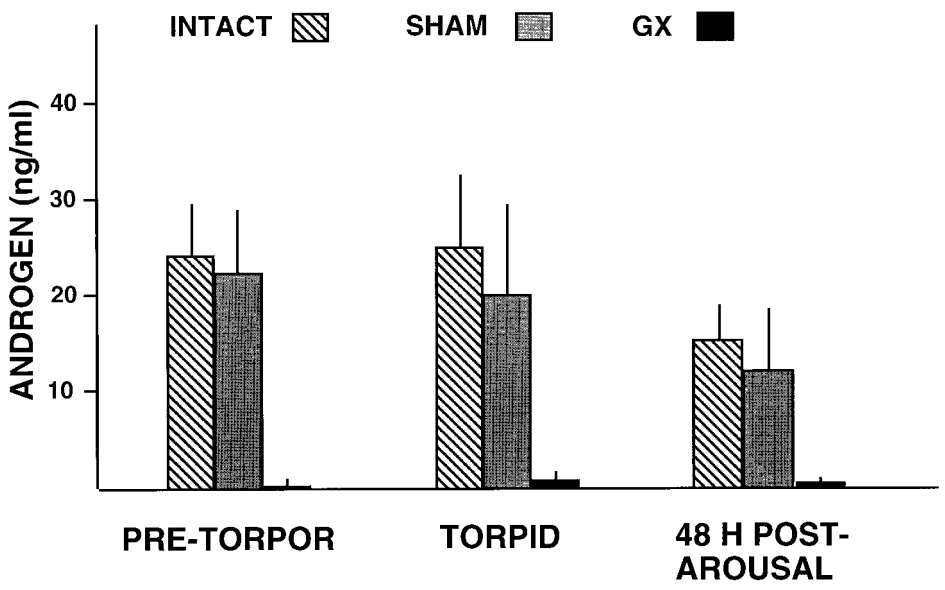

Figure 4. Androgen levels of intact (*INTACT, N* = 7), sham-operated (*SHAM, N* = 5), and gonadectomized (*GX, N* = 6) male big brown bats 4 wk before exposure to 4°C (pretorpor), after 6 d of exposure to 4°C (torpid), and 48 h after removal from 4°C (postarousal).

repertoire of copulatory behavior, although the frequency of the behavior was not as great as in our earlier study (Mendonça et al. 1996). There also was no correlation between intensity of mating activity (e.g., number of mounts) and androgen level.

It appears that previous testosterone exposure is necessary to initiate the neural changes that activate the behavior in the autumn. Once activated, reliance on circulating levels of testosterone decreases. Our ability to stimulate mating in otherwise nonmating individuals, regardless of presence of a gonad, by temperature manipulation alone suggests that other factors associated with arousal from hibernation may prove the more proximate activators.

Wimsatt (1960) broached the seeming paradox of overlapping periods of reproduction and hibernation in temperate bats. It may be that, in the big brown bat, at least at more southern latitudes where alternating periods of low and high temperatures are the norm in winter, these are not opposing physiological mechanisms but, rather, synergistic in relation to the expression of mating behavior. Current data indicate that activation of sexual behavior in male big brown bats may be seasonally organized by androgen and its effect potentiated, in the short-term, by changes in heterothermic state.

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