Annual cycle in LH and testosterone release in response to GnRH challenge in male woodchucks (*Marmota monax*)

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Testosterone and LH concentrations were determined in serum samples obtained before and 15 min after injections of GnRH ($1 \mu\text{g kg}^{-1}$) administered at 4–7 week intervals over 20 months to groups of male woodchucks ($n = 6\text{--}7$) born and maintained in Northern Hemisphere (boreal) versus Southern Hemisphere (austral) simulated natural photoperiods, beginning at 18–24 months of age. Nadir and peak unstimulated testosterone (0.1 ± 0.01 and $7.0 \pm 0.1 \text{ ng ml}^{-1}$, respectively) and LH (0.8 ± 0.2 and $8.1 \pm 1.1 \text{ ng ml}^{-1}$, respectively) concentrations did not differ in boreal versus austral males. In the five boreal and five austral males that were confirmed to be photoentrained, basal (pre-GnRH) concentrations of LH and testosterone were lowest in summer, increased simultaneously in late autumn or early winter, and declined in the spring. GnRH stimulated some LH release throughout the year except for a 1–4 month period in the summer. The initial annual increase in the LH response to GnRH occurred in early autumn, and in 17 of 20 cycles it occurred 1–2 months before the initial increase in basal LH was detected. In the three free-running males not entrained to the photoperiod, the endocrine patterns were similar but were advanced by several months. The results demonstrate that in woodchucks there is a late autumn increase in LH secretion associated with the onset of testicular recrudescence, and an early autumn increase in pituitary response to GnRH before a detectable increase in serum testosterone.

Introduction

Male woodchucks have a brief 1–2 month breeding season in late winter after a 2 month period of testicular recrudescence in late autumn and early winter (Christian *et al.*, 1972; Concannon *et al.*, 1990, 1996; Sinha Hikim *et al.*, 1991). Testicular recrudescence is initiated during subterranean hibernation in the wild, and shortly before the winter solstice in laboratory woodchucks maintained in simulated natural photoperiods (Concannon *et al.*, 1997). The breeding season is characterized by enlarged scrotal testes, production of fertile ejaculates (Concannon *et al.*, 1996), progressive regression of seminiferous tubules (Christian *et al.*, 1972), and peak or declining serum concentrations of testosterone (Baldwin *et al.*, 1985). The breeding season is followed by further testicular regression throughout the spring during a period of increased thyroid hormone activity (Christian *et al.*, 1972; Young *et al.*, 1986; Concannon *et al.*, 1993a). The testes become fully regressed and abdominal in summer and early autumn, a period characterized by low thyroid hormone activity and an increase in body weight to an annual peak. The extent to which cycles of testicular recrudescence and regression in woodchucks are related to seasonal changes in pituitary gonadotrophin secretion and serum gonadotrophin concentrations is not known. Circannual changes in testosterone, thyroid hormone and prolactin have been reported (Young *et al.*, 1986;

Concannon *et al.*, 1993a,b, 1995), but there are no data on circannual patterns of circulating gonadotrophin or on pituitary capacity to secrete gonadotrophin.

Woodchucks and several other species of marmotine sciurid rodents have endogenous circannual cycles that are unique. In the absence of photoperiodic cues, most woodchucks maintain circannual changes in gonadal activity, food intake and body weight with a free-running endogenous rhythm very close to 1 year for 1–3 years before reverting to free-running endogenous cycles with 9–10 month intervals (Pengelley and Asmundson, 1980; Concannon *et al.*, 1992). In other mammalian species, the free-running pattern is often highly variable and is usually much less than 12 months (Turek and Van Cauter, 1994). Woodchucks and related species are also unusual in the lighting cues required for photoentrainment. Block changes in photoperiod do not readily affect circannual cycles in these sciurid species (Davis, 1967, 1976; Pengelley and Asmundson, 1980). However, recent studies have demonstrated that daily changes in photoperiod that simulate natural photoperiods are sufficient, in the absence of other cues, to entrain the endogenous cycle of woodchucks to 12 months. Furthermore, maintenance of woodchucks in Southern Hemisphere (austral) photoperiods can advance the annual cycle by 6 months relative to that in animals in Northern Hemisphere (boreal) photoperiods, without changes in ambient temperature or the availability of food (Concannon *et al.*, 1993b, 1997).

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In the present study, groups of male woodchucks born and maintained in boreal versus austral photoperiods were studied to determine whether there are seasonal changes in basal concentrations of LH or in LH release provoked by a standardized GnRH challenge. The austral versus boreal photoperiod paradigm was used to ensure that changes observed over time were seasonal and were not related to potential changes in methodology or in environmental factors other than photoperiod (Concannon *et al.*, 1993b, 1997).

Materials and Methods

Animals and treatments

Male woodchucks were born and maintained in simulated natural photoperiods of Northern Hemisphere (boreal) latitude ($n = 7$) or Southern Hemisphere (austral) latitude ($n = 6$), and were studied over a 20 month period starting at 18–24 months of age. The photoperiodic changes approximated those of 42°N and 42°S, respectively. Daily photoperiod was provided by fluorescent lighting

(600–700 lux) regulated by computer controlled timers as previously described (Concannon *et al.*, 1993b, 1997). This involved changing the photophase by 1, 2, 3 or 4 min per day to yield solstices of 15 or 9 h of light per day on 20–21 June and 20–25 December, and equinoxes on 20–25 March and 20–25 September (Figs 1 and 2). Room temperatures were maintained at 20–23°C. At intervals of 4–7 weeks, animals were anaesthetized and blood samples (3–5 ml) were collected in evacuated tubes via inguinal venepuncture of the femoral vein at 0 and 15 min after injection of GnRH ($1 \mu\text{g kg}^{-1}$ i.m.). Samples were collected at 4 week intervals when austral males were expected to undergo testicular recrudescence and boreal males were undergoing testicular regression. Less frequent sampling at 5–7 week intervals was used at other times to reduce the potential effects of repeated GnRH administration, handling and anaesthesia on reproductive cycles. Anaesthesia was via intramuscular injection of ketamine ($50 \mu\text{g kg}^{-1}$; Fort Dodge Animal Health; Fort Dodge, IA) and xylazine ($5 \mu\text{g kg}^{-1}$; Bayer Corp., Shawnee Mission, KS). Blood samples were allowed to clot for 2 h, centrifuged for 30 min at 900 g , and serum was harvested and stored in aliquots at -20°C . Morphological

Fig. 1. (a) Daily photophases (----) and daily rate of change in photoperiod (—) used for photoentrainment, and mean ($\pm$ SEM) (b) serum LH and (c) testosterone before ($\circ$, $\square$) and after ($\bullet$, $\blacksquare$) injections of GnRH ($1 \mu\text{g kg}^{-1}$ i.m.) in five male woodchucks, *Marmota monax*, born and maintained in Northern Hemisphere (boreal) photoperiods. The animals remained photoentrained throughout the study.

Fig. 2. (a) Daily photophases (---) and daily rate of change in photoperiod (—) used for photoentrainment, and mean ($\pm$ SEM) (b) serum LH and (c) testosterone before (○, □) and after (●, ■) injections of GnRH ($1 \mu\text{g kg}^{-1}$ i.m.) in five male woodchucks, *Marmota monax*, born and maintained in Southern Hemisphere (austral) photoperiods. The animals remained photoentrained throughout the study.

recrudescence of the testis is initiated intra-abdominally and can be crudely monitored initially by abdominal palpation, and later by scrotal palpation (Baldwin *et al.*, 1985; Concannon *et al.*, 1993b). At the time of each GnRH challenge, the presence or absence of scrotal testes was noted, but testis size was not measured.

Hormone assays

Each serum sample was assayed for LH and testosterone content by radioimmunoassay. Testosterone was assayed using a commercial solid-phase kit (DPC Testosterone Coat-A-Count RIA, Diagnostics Products Corp., Los Angeles, CA), but with standards prepared in serum of castrate woodchucks and as reported by Concannon *et al.* (1993b). The limit of detection (95% binding) was 0.1 ng ml^{-1} . The intra- and interassay coefficients of variation for two serum samples included in each assay averaged 4 and 9%, respectively. LH was assayed using a heterologous assay validated and reported for use in dogs (Concannon, 1993). The LH assay reagents included a rabbit anti-rat LH serum

(CSU-120), highly purified ovine LH (LER-1056-C2) labelled with ^{125}I using chloramine-T and used as a tracer, and ovine LH (NIH-LH-S18) used as standard. The percentage crossreactivity of the anti-rat LH serum, based on 100% for rat LH and corrected for LH contamination of reference preparations, was 2.5% for rat thyroid stimulating hormone, 0.3% for rat FSH, 0.1% for rat prolactin, and 100% for ovine LH (G. Niswender, personal communication). Serial dilutions of sera from intact woodchucks ($n = 3$) and gonadectomized woodchucks ($n = 2$) yielded dilution curves that were not different ($P > 0.80$) from those obtained with standard hormone. The limit of detection (95% binding) was 0.5 ng ml^{-1} . For three serum samples included in each assay, the intra- and interassay coefficients of variation averaged 7 and 13%, respectively.

For each animal, the average of the lowest concentrations of LH or testosterone at three sequential time points was considered to be the mean nadir concentration for each hormone. The first increases after the nadir that were twice the limit of detection in LH ($\geq 1 \text{ ng ml}^{-1}$ above mean nadir) and in testosterone ($\geq 0.2 \text{ ng ml}^{-1}$ above mean nadir) were considered the first increases of that annual cycle. Increases

in LH $> 1.0 \text{ ng ml}^{-1}$ above the concentrations observed before injection were considered to be responses to GnRH; declines in LH and increases of $< 0.8 \text{ ng ml}^{-1}$ were considered non-responses as there were no changes between 0.8 and 1.0 ng ml^{-1} . Means are reported $\pm \text{SEM}$. Differences between and within groups in peak or nadir hormone concentrations or times of hormone increases were determined by Student's *t* test. Times of initial annual increases in mean hormone concentration or in responses to GnRH above the lowest annual value were determined by paired *t* tests. For each photoperiod group, the experimental intervals involving photoperiod changes corresponding to those naturally occurring in the Northern Hemisphere from 21 December to 20 March are referred to as winter, from 21 March to 20 June as spring, from 21 June to 20 September as summer, and the remainder as autumn.

Results

On the basis of observations of enlarged scrotal testes in winter and spring, entrainment to photoperiod during the study was confirmed in five of the seven males exposed to the boreal photoperiods, and in five of the six males exposed to austral photoperiods. The remaining three males were considered to be free running, based on the development of enlarged scrotal testes beginning in late summer or early autumn. Results for the free-running animals are considered separately. Among the photoentrained males, means for annual peaks for each parameter in each group were based on the average of the two available values for each of the animals.

Photoentrained males

Seasonal changes in mean basal serum concentrations of LH and testosterone, and of GnRH-stimulated LH and testosterone, were similar in photoentrained animals of both photoperiod groups (Figs 1 and 2). In both boreal and austral woodchucks, and in each year, mean testosterone concentration was lowest ($< 0.1 \pm 0.1$ to $0.1 \pm 0.1 \text{ ng ml}^{-1}$) in summer and highest (2.5 ± 0.6 to $7.3 \pm 2.0 \text{ ng ml}^{-1}$) in late winter. Nadir testosterone in individual males occurred in late summer and early autumn, ranging from non-detectable (< 0.05 – 0.14 ng ml^{-1}) to 0.15 ng ml^{-1} , and averaged $0.06 \pm 0.01 \text{ ng ml}^{-1}$ ($n = 20$). For 15 complete annual excursions studied in the ten photoentrained males, testosterone concentration was increased $\geq 0.3 \text{ ng ml}^{-1}$ for 4.4 ± 0.2 months (range 3–5 months). Initial increases in testosterone $\geq 0.3 \text{ ng ml}^{-1}$ were observed in late autumn or early winter, that is, in December or January in boreal males and in June or July in austral males. Peak testosterone in individual cycles ranged from 1.6 to 14.7 ng ml^{-1} , and averaged $7.0 \pm 0.9 \text{ ng ml}^{-1}$ ($n = 20$). Peak testosterone did not differ between the boreal ($7.9 \pm 1.3 \text{ ng ml}^{-1}$) and austral ($6.1 \pm 0.9 \text{ ng ml}^{-1}$) groups. Increases in testosterone in response to GnRH were generally limited to times when basal testosterone was increased above its nadir. The increases in testosterone were routinely greater at the middle than at the start or end of the breeding season. Peak

concentrations after GnRH did not differ between boreal ($13.7 \pm 1.8 \text{ ng ml}^{-1}$) and austral ($12.8 \pm 3.7 \text{ ng ml}^{-1}$) males.

In both groups of photoentrained males, mean serum LH concentrations were lowest ($0.8 \pm 0.2 \text{ ng ml}^{-1}$) in mid- and late summer and increased ($P < 0.05$) above nadir values in winter and early spring, beginning in January in boreal males and in late May or June in austral males. Mean LH was highest (4.2 ± 0.9 to $11.0 \pm 0.6 \text{ ng ml}^{-1}$) in winter or early spring (Figs 1 and 2). In individual cycles, peak LH concentration ranged from 3.4 to 16.2 ng ml^{-1} , averaged $8.1 \pm 1.1 \text{ ng ml}^{-1}$ and did not differ between boreal ($8.4 \pm 1.2 \text{ ng ml}^{-1}$, $n = 10$) and austral ($6.9 \pm 1.1 \text{ ng ml}^{-1}$, $n = 10$) groups. In individual animals, nadir LH concentrations ranged from < 0.5 to 1.2 ng ml^{-1} and averaged $0.7 \pm 0.1 \text{ ng ml}^{-1}$. In individual males, initial increases in basal LH $\geq 1.0 \text{ ng ml}^{-1}$ above nadir reached concentrations of $2.5 \pm 0.9 \text{ ng ml}^{-1}$, and occurred in late autumn or early winter, that is, in December or January in boreal males, and in June or July in most austral males. In 18 of 20 cases, the annual increases in LH were coincident with initial increases in testosterone. In one case, there was a distinct increase in LH before the first increase in testosterone, and in one case testosterone appeared to increase first. The mean interval between the first detected increase in LH and that of testosterone was 0.0 ± 0.1 months ($n = 20$).

Mean serum LH concentration after injection with GnRH was lowest (1.3 ± 0.2 to $2.8 \pm 1.2 \text{ ng ml}^{-1}$) in the summer, and increased ($P < 0.05$) above the lowest values in late autumn, beginning in November and December in boreal males, and in April in austral males. Mean values for LH concentration after GnRH increased in winter and spring, and were highest (19 ± 3 to $28 \pm 6 \text{ ng ml}^{-1}$) in late winter (Figs 1 and 2). Maximal LH responses to GnRH in individual cycles ($25 \pm 1.4 \text{ ng ml}^{-1}$) occurred in late winter and early spring in both groups, and were similar in the boreal ($24 \pm 2 \text{ ng ml}^{-1}$) and austral ($25 \pm 3 \text{ ng ml}^{-1}$) males. In individual cycles, LH concentrations after GnRH were lowest in late summer, ranging from < 0.5 to 2.2 ng ml^{-1} , and averaging $1.6 \pm 0.4 \text{ ng ml}^{-1}$. Furthermore, in individual cycles, LH did not increase in response to GnRH for 1–4 months during the summer and early autumn. Subsequent initial increases in the LH response to GnRH reached values of $3.8 \pm 1.2 \text{ ng ml}^{-1}$ and usually occurred in autumn, that is, in September–December in boreal animals and in April or May in austral animals. The initial seasonal increase in the amount of LH released in response to GnRH occurred 1–2 months before the first detected increase in basal LH or testosterone in 17 of 20 instances. For the 20 breeding seasons initiated during the study, the initial response to GnRH occurred 1.2 ± 0.2 months before ($P < 0.05$) the first observed increase in basal LH or testosterone. Major declines in basal LH and testosterone occurred in the spring, while LH responses to GnRH remained high albeit below maximal (Figs 1 and 2).

Free-running males

Three males (two boreal and one austral) had circannual increases in LH and testosterone associated with scrotal testes at intervals of about 8–10 months, similar to those of

free-running males in other studies (Concannon *et al.*, 1997). On the basis of these observations, and on subsequent observations, the three males were considered to be free running and not photoentrained. For the second breeding season of the two free-running males studied in the boreal photoperiod, basal LH and testosterone concentrations increased in September or November, in contrast to January or February in the photoentrained boreal males (Fig. 3). In one of the free-running boreal males, a third increase in testosterone was initiated in April, approximately 8 months after the previous initial increase in testosterone (Fig. 3). In the free-running austral male, basal LH and testosterone increased in April, in contrast to June or July in the photoentrained austral males.

Discussion

The patterns of testosterone in the animals in this study were similar to those previously observed in woodchucks maintained in boreal and austral photoperiods (Concannon *et al.*, 1993b, 1997). The patterns of serum LH observed in this study suggest that an increase in LH secretion is associated with the onset of testicular recrudescence in woodchucks, as reflected in serum testosterone concentrations. Presumably,

the initial increase in LH is an element of the endogenous circannual cycle that is entrained by patterns of daily photoperiod. Circannual changes in LH associated with testicular regression and recrudescence have been reported in a related sciurid rodent, the golden mantled ground squirrel (Licht *et al.*, 1982; Barnes, 1986). In ground squirrels, concentrations of LH and testosterone increase at the same time in early winter, peak in late winter, and decline thereafter, with nadir values in late summer and autumn, even when the animals are maintained in a constant photoperiod (Barnes, 1986). This is similar to the results obtained for the woodchucks in the present study. In some mammalian species, annual changes in LH secretion associated with the breeding seasons are due, at least in part, to an altered hypothalamic-hypophyseal sensitivity to gonadal steroid negative feedback (Legan *et al.*, 1977; Tamarkin *et al.*, 1977; Robinson *et al.*, 1985; Lubbers and Jackson, 1993; Woodfill *et al.*, 1994). The same may be true for woodchucks and other marmotine rodents.

The seasonal increases in GnRH-induced LH release were usually observed 1–2 months earlier than increases in basal LH and testosterone were detected. These early responses could be due to changes in releasable amounts of LH, pituitary sensitivity to GnRH, or both. The absence of concomitant increases in basal LH release could be the result

Fig. 3. Basal serum testosterone (□), and basal (○) and GnRH-stimulated (●) LH, and periods of enlarged scrotal testes (bars) in (a,b) two free-running woodchucks and (c) a photoentrained woodchuck (*Marmota monax*). All three animals were born and maintained in Northern Hemisphere (boreal) photoperiods.

of a deficiency in endogenous GnRH until shortly before the onset of testicular recrudescence. Alternatively, small and transient increases in endogenous LH may have occurred at that time, and before initial increases in testosterone, but were not detected in the present study due to infrequent sampling or the sensitivity of the assay. The use of standardized GnRH challenges as applied in the present study is a useful method for evaluating seasonal changes in the combined functional state of the tissues regulating LH secretion in this species.

The temporal relationship between the decline in mean testosterone concentration, testis size, and LH concentrations suggests that a reduction in the availability of LH may be involved in the initiation of testicular regression and the termination of the breeding season. Additional factors are likely to be involved, because regressive changes in the seminiferous tubules are evident even at the start of the brief breeding season (Christian *et al.*, 1972). In some species, gonadal regression associated with refractoriness to an otherwise stimulatory photoperiod is dependent upon the availability of thyroid hormone. The fact that thyroid hormone activity increases before and during the period of declining testosterone activity and gross testicular regression in woodchucks (Concannon *et al.*, 1993a, 1995) may be relevant.

The mechanisms by which changes in photoperiod entrain various elements of the endogenous circannual rhythm in woodchucks are not known. The present findings demonstrate an initial increase in GnRH-induced LH release in the early autumn, when the photoperiod is decreasing, and increases in spontaneous gonadotrophin secretion in late autumn before the shortest day of the year. Such temporal relationships suggest that, in this species, decreasing or short days are stimulatory and that lengthening or long days are inhibitory to the endogenous mechanisms regulating the pituitary–gonadal axis. However, seasonal photorefractoriness has been suggested as a mechanism for initiation and termination of the breeding season in both long-day and short-day breeders (Malpaux *et al.*, 1989; Martinet *et al.*, 1992). In woodchucks, it is possible that during the autumn and winter the increases in pituitary responsiveness and in spontaneous gonadotrophin secretion occur because critical hypothalamic–hypophyseal elements become refractory to inhibitory influences previously provided by shortening or short days. Similarly, decreases in gonadotrophin secretion in the spring could involve animals becoming refractory to previously stimulatory lengthening or long days. It is likely that lengthening or long days are stimulatory to gonadal function in woodchucks. In males maintained in an 8 month circannual photoperiod cycle, premature gonadal recrudescence occurs during days that are prematurely lengthened (Concannon *et al.*, 1996).

Whether the observed time course of testicular recrudescence and the presumed role of photorefractoriness observed in laboratory woodchucks also apply to recrudescence in hibernating woodchucks in constant darkness is not known. Woodchucks maintained in simulated natural photoperiods typically breed and give birth about 3–4 weeks earlier than those in the wild (Concannon *et al.*, 1997). It is possible that initiation of

testicular recrudescence during hibernation occurs at the same time in the wild as in the laboratory, but that the process of recrudescence is slower in the wild because of the metabolic effects of hibernation.

The present results suggest that seasonal changes in LH secretion have a role in the timing and process of testicular recrudescence in woodchucks. Presumably, seasonal changes in serum FSH also occur. Barnes (1986) reported seasonal changes in FSH in ground squirrels. The data for the three free-running males are consistent, within individuals, with data for the photoentrained animals, including simultaneous detectable increases in basal LH and testosterone that are preceded by 1 or more months of increased capacity for LH release in response to exogenous GnRH. In these animals, as in free-running woodchucks observed under similar conditions (Concannon *et al.*, 1997), it is not clear whether the free-running phenomenon represents individual abnormalities, the extreme of a range of normal responses, or an inability to respond to a light intensity that might be marginal for photoentrainment in this species.

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