Annual changes in serum concentrations of prolactin in captive male black bears (*Ursus americanus*)

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Prolactin may be involved in the regulation of reproduction in black bears (*Ursus americanus*) as it is a mediator of photoperiodic changes in a number of species. The objectives of this study were to validate a radioimmunoassay to measure prolactin in bear serum and to describe seasonal changes in serum prolactin concentrations in captive male black bears. Serum samples were obtained nine times during a year from three captive male black bears that were denning between November and March and active during the other months. The heterologous prolactin radioimmunoassay, using pig ¹²⁵I-labelled prolactin and goat anti-pig prolactin as a primary antibody, was validated. Injection of thyrotrophin-releasing hormone into the three male bears in June resulted in a rapid increase in serum concentrations of prolactin ($t = 0$, 11.4–14.8 ng ml⁻¹; $t = 15$ –30 min, 18.4–28.7 ng ml⁻¹). The sensitivity of the assay was 0.08 ng per tube. Intra- and interassay coefficients of variation were 5.5% ($n = 6$) and 5.7% ($n = 6$), respectively. Serum concentrations of prolactin changed seasonally, with the lowest concentrations in December (mean $\pm$ SD = 1.1 $\pm$ 0.1 ng ml⁻¹); this was followed by a gradual increase between January (2.6 $\pm$ 0.6 ng ml⁻¹) and April (6.4 $\pm$ 1.2 ng ml⁻¹) and the highest concentrations in May (17.6 $\pm$ 4.7 ng ml⁻¹), preceding peak testosterone concentrations in June. The observation that prolactin secretion increased with increasing daylength suggests that photoperiod may be an external regulator. The presence of high concentrations of prolactin before peak testosterone concentrations suggests that prolactin may play a role in regulating seasonal changes in the testes.

Introduction

American black bears (*Ursus americanus*) have a mating season from late spring to early summer (Erickson and Nellor, 1964; Reynolds and Beecham, 1980) and den during the winter season (Nelson *et al.*, 1973). Seasonal changes in serum testosterone and LH concentrations were reported in male black bears (McMillin *et al.*, 1976; Palmer *et al.*, 1988; Tsubota and Kanagawa, 1989; Horan *et al.*, 1993). Profiles of serum FSH and prolactin have not been reported. Immunocytochemical localization of steroid enzymes in the testes of black bears indicated that spermatogenesis is initiated during the mid-denning period (January) and is associated with a rise in serum testosterone and oestradiol concentrations. These steroids may be synthesized in Leydig cells and Sertoli cells, respectively (Tsubota *et al.*, 1993). However, it is not known what

stimulates and maintains the steroid synthesis associated with spermatogenesis in the bear testis.

Prolactin is a pituitary hormone that changes in response to photoperiod. In general, long days increase prolactin concentrations whereas short days decrease secretion (Pelletier, 1973; Lincoln *et al.*, 1978). Prolactin plays a major role in seasonal changes in reproduction (Curlewis, 1992), pelage moult cycle (Duncan and Goldman, 1984; Smith *et al.*, 1987; Curlewis *et al.*, 1988), growth and metabolism (Eisemann *et al.*, 1984; Bartness *et al.*, 1987; Curlewis *et al.*, 1988). However, its precise role in male reproduction in some mammalian species is controversial. In sheep, the annual change in prolactin concentration is not causally linked to the seasonal pattern of gonadal activity (Barenton and Pelletier, 1983), whereas increasing prolactin concentrations in spring may be important for reproduction in golden hamsters (Klemcke *et al.*, 1984; Bartke *et al.*, 1984) and blue foxes (Smith *et al.*, 1987).

The objectives of this study were to establish a prolactin assay for bear serum using a porcine prolactin radioimmunoassay and to describe seasonal changes in serum prolactin concentrations in captive male black bears.

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Materials and Methods

Animals

Three adult male American black bears, aged 6–8 years, were used in this study. These bears were sexually mature because their testes exhibited full spermatogenesis during the mating season (Tsubota *et al.*, 1993). They were housed in a United States Department of Agriculture (USDA) approved facility, consisting of an outdoor run and denning area. All studies were approved by The Carle Foundation Animal Care and Use Committee. Commercial dog chow (18% protein, 6% crude fibre, 6% crude fat) and water were provided until denning. Bears entered dens around 13 October 1992 and left them on or around 12 March 1993. Bears were subjected to natural photoperiod throughout the year. Water was available throughout denning, but the bears seldom drank.

Blood sampling

For all studies, bears were first anaesthetized i.m. with Telazol (Elkins-Sinn Inc., Cherry Hill, NJ) at a dose of 5 mg kg⁻¹ body mass, using a metallic poke stick syringe. Telazol contains tiletamine hydrochloride and zolazepam hydrochloride. Blood was sampled from the femoral vein at 15–20 min intervals, when repeated sampling was necessary. Immediately after collection, blood was centrifuged at 1200 g for 20 min at 4°C, and the serum was removed and stored at –20°C until assayed.

Behaviour of bears

The bears demonstrated the usual behaviour associated with seasonal changes. In summer, food intake was 5000–8000 kcal day⁻¹ (Nelson *et al.*, 1983). In autumn, hyperphagia occurred, with food intake approaching 20 000 kcal day⁻¹. With the onset of denning, aphagia occurred and persisted throughout the five month period of denning. Bears rarely left their dens to drink water. They were alert when approached and required similar amounts of anaesthetic to active bears. The serum urea:creatinine ratio decreased to values of ten or less, which is indicative of the metabolic adaptation to denning (Nelson *et al.*, 1984).

Prolactin assay

A heterologous radioimmunoassay using pig ¹²⁵I-labelled prolactin and goat anti-pig prolactin serum as a primary antibody was established. Purified porcine prolactin (USDA-PRL-B-1) for iodination and standards and primary antiserum were obtained from D. J. Bolt (USDA Animal Hormone Program). The following procedure was used with modifications: 0.05 mol phosphate buffer l⁻¹, pH 7.4 (25 µl) was added to the reaction microtube containing pig prolactin (5 µg). ¹²⁵I-Na (0.05 mCi) (Amersham Corporation, Arlington Heights, IL) was added to the tube. The solution was mixed with 0.5% (w/v) chloramine-T (5 µl) for 60 s and then 0.1% (w/v) sodium metabisulfite (20 µl) was added for 2–5 s. After 0.05 mol phosphate buffer l⁻¹ (200 µl) was added to the tube, the

contents of the tube were drawn into a 1 ml disposable syringe and placed on a column that contained approximately 0.5 ml anion exchange resin (Bio-Rad-Analytical Grade Anion Exchange Resin AG1-X10, 100–200 mesh). The solution was collected from the column into a tube containing 0.5 ml assay buffer (PBS, containing 1% (w/v) BSA, pH 7.4). The binding of the ¹²⁵I-labelled prolactin was checked by reacting 10 000 c.p.m. with several different concentrations of the primary antibody. The optimal binding of the primary antibody was approximately 30% binding.

On the first day, standards and samples (200 µl) plus assay buffer (400 µl) were incubated with primary antibody (1:30 000; 100 µl) and ¹²⁵I-labelled prolactin (approximately 10 000 c.p.m. in 100 µl) at 4°C for 48 h. On the third day, the second antibody (rabbit anti-goat gamma globulin 1:15; Antibodies Incorporated, Davis, CA) and 6% polyethylene glycol (6000 molecular weight) were added to the tubes and they were centrifuged at 1500 g at 4°C for 30 min. The supernatant was removed by aspiration and the radioactivity of the pellet was measured in a gamma counter. Each sample was assayed in duplicate. Tubes containing normal goat serum instead of primary antibody were treated by the same procedure to determine nonspecific binding. A pooled sample was assayed at two different volumes in each assay. Intra- and interassay coefficients of variation were 5.5% (*n* = 6) and 5.7% (*n* = 6), respectively.

Validation of the prolactin assay

The pig prolactin radioimmunoassay was validated according to the following steps. First, increasing amounts of pooled bear sera and pituitary homogenates were assayed to determine parallelism. The pituitary was obtained from a dead bear, stored at –80°C, homogenized using a sonicator and diluted 1:10 before assay. Second, three anaesthetized male black bears were injected i.v. with 7 µg thyrotrophin releasing hormone (TRH) kg⁻¹ body mass and blood was sampled before TRH injections and at intervals of 15 min thereafter for 2 h. This experiment was done in June when bears were reproductively active. Sera were stored at –20°C until assayed for prolactin.

Testosterone assay

Serum concentrations of testosterone were measured by radioimmunoassay as described by Palmer *et al.* (1988). Double-extraction with anaesthesia grade ether and a mixture of 75% (v/v) methanol and hexane was followed by radioimmunoassay. Mean recovery rate after extraction was 83.2%. All samples were corrected for procedural losses. The crossreactivity of the antiserum was reported by Bahr *et al.* (1983). The testosterone assay for black bear serum was validated by column chromatography, parallelism and recovery of unlabelled ligand (Palmer *et al.*, 1988). The sensitivity of the testosterone assay was 15 pg. Intra- and interassay coefficients of variation were 7.7% (*n* = 5) and 13.3% (*n* = 9), respectively. Statistical analysis was carried out by Student's *t* test.

Fig. 1. Standard curve with pig prolactin and different amounts of prolactin in the bear serum and pituitary homogenate for three individual bears. This graph shows the parallelism between the standard curve and increasing amounts of prolactin in the bear serum (—) and pituitary homogenate (---). Parallelism was assessed by regression analysis and comparison of the slopes by *t* test.

Results

Validation of the prolactin assay

A plot of values obtained for several dilutions of bear serum (10–200 μ l) but not for the pituitary homogenate (0.6–10 μ l) was parallel to the standard curve for pig prolactin (Fig. 1). Intravenous injection of three bears with 7 μ g TRH kg^{-1} body mass caused a significant increase ($P < 0.002$) in serum concentrations of prolactin from 11.4–14.8 ng ml^{-1} to 18.4–28.7 ng ml^{-1} within 15–30 min followed by a gradual decrease (Fig. 2).

Annual changes in serum testosterone and prolactin concentrations

Serum concentrations of testosterone were low during January (0.4–0.6 ng ml^{-1}), began to increase in February (bear 1) or March (bears 2 and 3) and peaked in June (12.4–63.2 ng ml^{-1}) (Fig. 3). Testosterone concentrations decreased to 0.1–2.0 ng ml^{-1} in October, and then increased ($P < 0.01$) in December (5.0–7.1 ng ml^{-1}). The lowest concentrations of prolactin (1.0–1.1 ng ml^{-1}) were measured in December (Fig. 4). Prolactin then increased gradually through February (2.0–2.9 ng ml^{-1}) and March (2.6–3.2 ng ml^{-1}). Serum prolactin concentrations increased further, from 4.3–6.3 ng ml^{-1} in March to 5.0–7.2 ng ml^{-1} in April and to high concentrations ($P < 0.02$) in May (12.4–21.6 ng ml^{-1}) preceding the testosterone peak in June. However, prolactin concentrations decreased again in June (12.2–13.7 ng ml^{-1}). The serum concentrations of prolactin of the three male bears were similar in amounts and in seasonal changes. In contrast, there were greater differences in serum concentrations of testosterone among the three bears; however, the seasonal changes were similar.

Fig. 2. Changes in serum concentrations of prolactin after i.v. injection of thyrotrophin releasing hormone (TRH) (7 μ g kg^{-1} body mass) into three male black bears during the mating season in June. *Averaged prolactin concentrations for the three bears were significantly higher than concentrations at time 0 ($P < 0.05$).

Fig. 3. Annual changes in serum testosterone concentrations in three male black bears. *Significant difference between the average testosterone concentration for the three bears and the subsequent average testosterone concentration ($P < 0.05$). The diagram at the top of the graph indicates the change in daylength with season. Clear area: daylight; black area: darkness.

Discussion

This is the first known report of serum prolactin measurements in black bears sampled at various times throughout a year; it demonstrates the validation of a pig prolactin radioimmunoassay to measure prolactin concentrations in bears. The prolactin assay was validated by two different methods. First, parallelism between the pig prolactin standard curve and increasing volumes of bear serum and pituitary extracts was found. Second, TRH injection of male bears with complete spermatogenesis (Tsubota *et al.*, 1993) caused a significant increase in prolactin release within 15 to 30 min. Similar increases in serum prolactin concentrations after TRH injection were reported in humans (Bowers *et al.*, 1971), cows and bulls

Fig. 4. Annual changes in serum concentrations of prolactin in three male black bears. *Significant difference between the average prolactin concentration for the three bears and the subsequent average prolactin concentration ($P < 0.05$). The diagram at the top of the graph indicates the change in daylength with season. Clear area: daylength; black area: darkness.

(Convey *et al.*, 1973), pigs (Dubreuil *et al.*, 1988; Stuber *et al.*, 1990), domestic dogs (Reimers *et al.*, 1978) and wolves (Kreeger *et al.*, 1991).

This study reports a seasonal change in serum concentrations of prolactin in black bears. Concentrations were lowest in December and increased gradually through February and March, reached peak values in May and then gradually declined. This circannual prolactin rhythm is similar to that of other mammals and appears to be mediated by photoperiod; concentrations decrease with decreasing daylength and increase with increasing daylength, although two bears exhibited a decline of prolactin release in June when daylength was still increasing. This change in prolactin concentrations correlates well with the regression of the bear testis in autumn and winter (Horan *et al.*, 1993) and its gradual recrudescence beginning in January and resulting in full spermatogenesis and steroidogenesis in spring (Tsubota *et al.*, 1993). This seasonal pattern of prolactin secretion and the presence of high serum prolactin concentrations one month before maximum serum testosterone concentrations suggests a role for prolactin in the regulation of seasonal testicular function in black bears.

Prolactin is known to be involved in the regulation of male testicular function. In golden hamsters, it appears to modulate the effects of photoperiod on gonadal function (Bartke *et al.*, 1975). If the golden hamster is used as a model to understand seasonal reproductive activity many possible functions can be suggested for prolactin in black bears. Foremost, it stimulates the activity of the testes of hamsters by increasing the number of LH receptors (Bex and Bartke, 1977). Second, prolactin increases the activity of the steroidogenic enzymes of the testis, 3β - and 17β -hydroxysteroid dehydrogenase (Hafiez *et al.*, 1971; Musto *et al.*, 1972). Third, it promotes the accumulation of testicular esterified cholesterol (Bartke, 1971). In addition, the increase in prolactin concentrations upon exposure to long photoperiods causes a decrease in the negative feedback sensitivity to testosterone (Matt *et al.*, 1984). Each of these physiological changes enhances testicular steroidogenesis, resulting in an active testis and perhaps the

annual serum testosterone peak. Localization of both prolactin and LH receptors in bear testes, either by immunocytochemistry or *in situ* hybridization will further elucidate the role of prolactin in seasonal reproduction of male black bears.

Serum concentrations of testosterone in black and brown bears are highest in spring and lowest in autumn, a few months before denning (McMillin *et al.*, 1976; Palmer *et al.*, 1988; Tsubota and Kanagawa, 1989). While these studies reported the testosterone peak of free ranging bears to be in April or May, testosterone concentrations in the captive bears studied here were highest in June (Horan *et al.*, 1993; Tsubota *et al.*, 1993). In addition, the results of this study showed another testosterone peak in December, although its magnitude was considerably lower than the peak in June. According to morphological and immunocytochemical studies of spermatogenesis and steroidogenic enzymes in bear testes, spermatogenic activity is initiated in January, the mid-denning time (Tsubota *et al.*, 1993). This finding suggests that the small peak in serum testosterone in December is associated with the initiation of spermatogenesis in January. Testosterone has been shown to reinstate spermatogenesis in adult stalk-sectioned Rhesus monkeys (Marshall *et al.*, 1983) and hypophysectomized rats (Huang *et al.*, 1987).

In summary, a valid pig prolactin radioimmunoassay for bear serum has been described, seasonal changes in serum prolactin concentrations have been reported, and a second, smaller serum testosterone peak in December has been identified. These results suggest that prolactin plays a regulatory role in testicular recrudescence in black bears. On the basis of evidence from studies of golden hamsters, it is possible that prolactin is required during the latter part of denning to enhance testicular steroidogenesis and to prepare bears for the mating season in June. While the December rise in testosterone concentrations does not correlate with prolactin concentrations, it is possible that this testosterone peak is independently associated with the initiation of spermatogenesis. Further studies will elucidate the roles of both prolactin and testosterone in the regulation of seasonal reproduction in black bears.

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