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Changes in Serum Inhibin Levels and Immunolocalization of Inhibin/Activin Subunits During the Breeding Season in the Wild Male Japanese Black Bear (*Ursus thibetanus japonicus*)

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The objective of this study was to investigate the changes in secretion of inhibin and cellular localization of inhibin α and inhibin/activin (β_A and β_B) subunits during the breeding season in the wild male Japanese black bear. Histological observations of testes were performed and seminiferous tubule diameters were measured. The sections of the testes were immunostained by the avidin–biotin–peroxidase complex method (ABC) using polyclonal antisera raised against porcine inhibin α, inhibin/activin β_A, and inhibin/activin β_B during the breeding season. Serum concentrations of immunoreactive (ir-)inhibin, testosterone, and luteinizing hormone (LH) were measured by radioimmunoassay. Higher values of seminiferous tubule diameters and all types of spermatogenic cells including mature-phase spermatozoa were found during the breeding season. There were seasonal changes in serum concentrations of ir-inhibin, testosterone, and LH. Ir-inhibin was positively correlated with testosterone, and LH. In addition, immunoreactivity of inhibin α, β_A, and β_B subunits were also detected in Sertoli and Leydig cells during the breeding season. These results suggest that Japanese black bear testes may secrete bioactive inhibins during the breeding season and that the circulating inhibin may be a useful indicator of the testicular function in wild male Japanese black bears.

Key Words: Japanese black bear; inhibin; testis; testosterone; LH.

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Introduction

The inhibin/activin family of dimeric peptide hormones are produced in the testes and are postulated to have paracrine and autocrine roles in the regulation of steroidogenesis and spermatogenesis in addition to endocrine regulatory effects on FSH secretion *(1–4)*. Inhibin consists of an α subunit linked by a disulfide bridge to one of the two highly homologous β subunits (βA and βB) to form inhibin A (α and βA) or inhibin B (α and βB). The various inhibin/activin subunits have been localized in the testes as messenger RNA and proteins. In the stallion and rams, inhibin α, β_A, and β_B subunits were present in both Sertoli and Leydig cells *(5,6)*, and in adult monkey, all three subunits were localized predominantly in Sertoli cells *(7,8)*. The expression of mRNA in Sertoli cells varies with the stage of the seminiferous cycle *(9)*, with differences between the various subunits possibly reflecting differential production of activin and inhibin *(10)*. In the male, inhibin is produced in the testis, principally by the Sertoli cells. There are temporal changes in inhibin expression and secretion with the changing role of the Sertoli cell in immature and adult testes. Some seasonal breeders, such as stallions *(5)*, rams *(11)*, and Japanese monkeys *(8,12)*, showed seasonal changes in circulating inhibin concentrations in accordance with the testicular activity. However, those limited data showing seasonal changes in inhibin secretion appeared to be more prominent in experimental and domestic animals than in the wild animals.

The wild male Japanese black bear is a seasonal breeding species. Spermatogenesis and steroidogenesis are maximal before and during the breeding season, which extends from May to August, with peak breeding occurring in June *(13–17)*. Age of sexual maturation in the wild male Japanese black bears was estimated to be 3–4 yr and mature males show highly synchronized testicular cycles including their

weight, size, and diameter of seminiferous tubules *(17)*. Studies in the Japanese black bears have shown this annual cycle of involution and recrudescence of testes in connection with plasma concentrations of testosterone and spermatogenesis *(17)*. Although several observations about reproductive biology in the male Japanese black bears are known *(13–17)*, there are still many gaps in the understanding of the mechanisms of reproduction, especially the correlation among secretions of inhibin and other hormones as testosterone and LH. Also, the cellular sources of inhibin in wild male Japanese black bears have not yet been determined. Therefore, the aims of this study were to investigate the cellular localization of the inhibin α, β_A, β_B subunits during the breeding season and seasonal changes in serum concentrations of inhibin, testosterone and LH in wild male Japanese black bears.

Results

Histology

A number of seminiferous tubules and the interstitial connective tissue were observed in the testes examined. The most prominent cells in the interstitium were Leydig cells. Each Leydig cell had a large round nucleus and a large cytoplasm. The entire spermatogenic cell population from spermatogonia to spermatozoa was present in June (Fig. 1A). On the other hand, spermatogonia and spermatocytes were found in September (Fig. 1B).

Immunohistochemistry

Immunoreactivities for inhibin α and inhibin/activin (β_A and β_B) subunits were present in the testes of wild male Japanese black bears during breeding season (June). Inhibin α and inhibin/activin (β_A and β_B) subunits were expressed in the cytoplasm of Leydig and Sertoli cells in the breeding season, respectively (Figs.1C,D,E). Immunoreactivity for inhibin α and inhibin/activin (β_A and β_B) subunits was not observed in germ cells during the breeding season. No immunostaining was detected in control sections in which normal rabbit serum was substituted in place of the primary antibody (Fig.1F).

Diameter of Seminiferous Tubules

Seasonal changes were observed in the diameter of seminiferous tubules (Fig. 2). The values of the seminiferous tubule diameters were higher in June (207.9 ± 7.7 μm) and July (201.7 ± 7.5 μm), began to decline in August (193.4 ± 13.5 μm), and thereafter exhibited their lowest values in September (99.3 ± 7.7 μm).

Concentrations of IR-Inhibin, Testosterone, and LH

Serum concentrations of ir-inhibin, testosterone, and LH are shown in Figs. 3A,B,C). There were significant changes in serum concentrations of ir-inhibin, testosterone, and LH from May to September. All hormones showed higher concentrations during the breeding season (June and July) and declined in August and September. The circulating concentration of ir-inhibin was positively correlated with testosterone ($r = 0.7$) and LH ($r = 0.7$).

Discussion

The present study demonstrated that seasonal changes in circulating inhibin, testosterone, and LH occurred in wild male Japanese black bears, and the peak levels of these hormones were found during the breeding season. Seasonal changes for spermatogenesis and seminiferous tubule diameters in male Japanese black bears were also observed and testicular activity is accompanied by changes in circulating inhibin, testosterone, and LH concentrations in this study. In addition, our immunohistochemical results demonstrated that inhibin α, β_A, and β_B subunits positive staining were observed in both Sertoli and Leydig cells in the breeding season. These results suggest that Sertoli cells and Leydig cells of the black bears could secrete bioactive inhibins in the breeding season, and that inhibin may be a useful marker of testicular function in wild male Japanese black bears.

Seasonal changes for serum inhibin levels in male Japanese black bears were first observed in this study, and it showed that serum inhibin concentrations were high in June and July and declined in August and September, which is not unique to Japanese black bears. Other seasonal breeders, such as stallions *(5,18)*, rams *(11)*, and Japanese monkeys *(12)*, showed similar seasonal changes in circulating inhibin concentration in accordance with the testicular activity. In the present study, seasonal changes in inhibin were positively correlated with those in testosterone and LH and the inhibin concentrations exhibited higher values during the breeding season. These results are in agreement with observations from previous studies in male bears. Serum levels of testosterone and accompanying testicular weight and size in male black bears were low during the nonbreeding season, and higher in breeding season *(17)*, and that serum testosterone concentrations first increased in March, coincident with increased testicular LH-receptor mRNA *(16)*. Likewise, immunocytochemical staining for 3β-hydroxysteroid dehydrogenase (3βHSD) and 17α-hydroxylase cytochrome P450 (P450c17) in the black bear testes increased dramatically between January and June *(13)*. Previous studies in other seasonal breeders, such as stallions *(5)* and golden hamsters *(19)*, also showed positive relationships between serum inhibin, LH, and testosterone concentrations suggesting that inhibin may be involved in spermatogenesis. Therefore, our results here showed that higher serum concentrations of inhibin were observed in the breeding season, which is in agreement with previous studies that the circulating inhibin may be a useful indicator of the testicular function, which plays a role in the regulation mechanism involved in spermatogenesis.

The distribution of inhibin subunits in the normal adult male corresponds to the sites of production of inhibins in

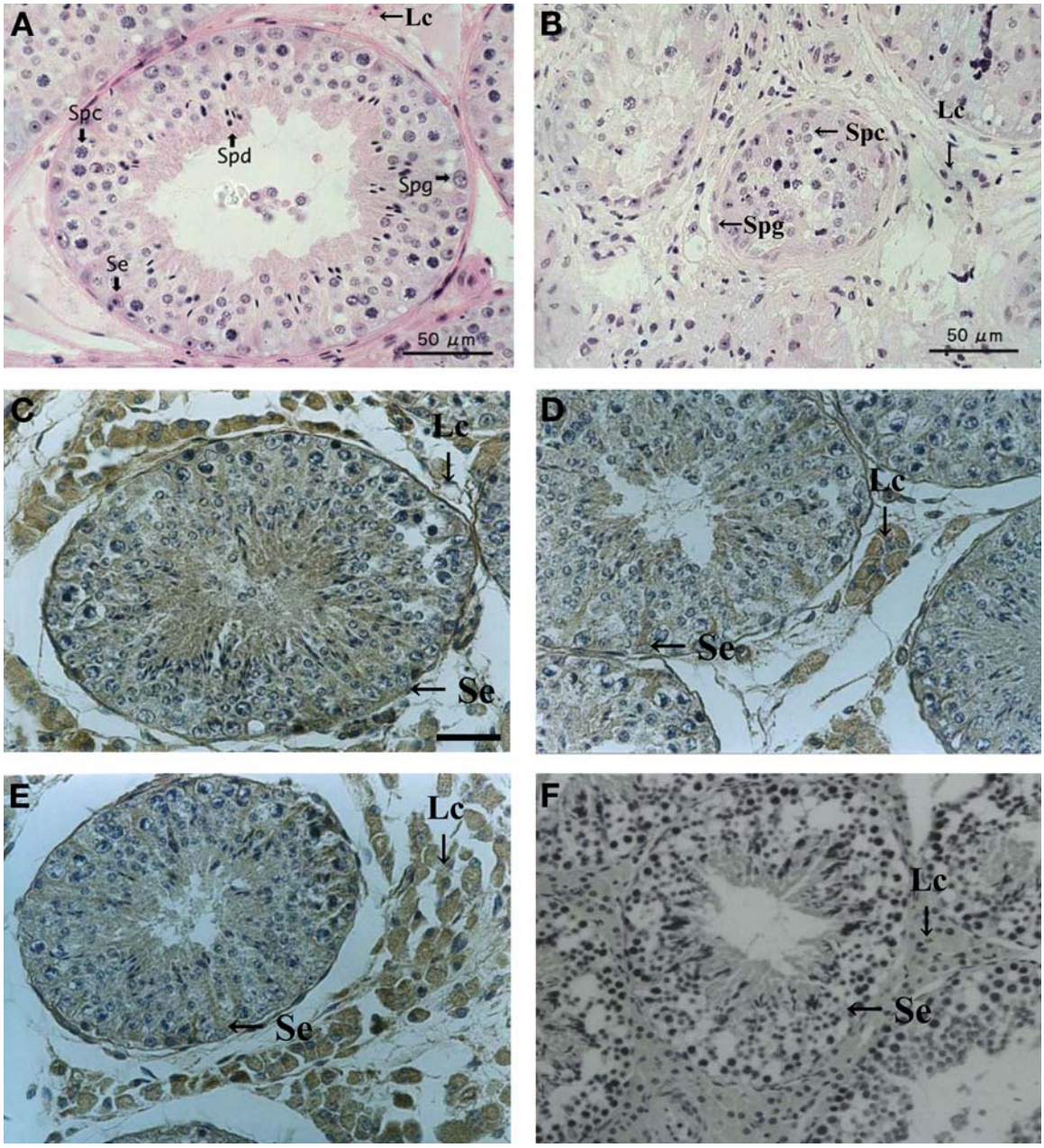

Fig. 1. Axial section of seminiferous tubules of the wild male Japanese black bear was stained by hematoxylin–eosin (**A,B**). Spermatogonia, spermatocytes, and spermatids were present in June (**A**). Spermatogonia and spermatocytes were observed in September (**B**). Immunolocalization of inhibin α and inhibin/activin (β_A and β_B) subunits in the wild male Japanese black bears testes during the breeding season (June) (**C,D,E,F**). Immunostaining for inhibin α and inhibin/activin (β_A and β_B) subunits were found in Leydig cells and Sertoli cells, respectively (**C,D,E**). Immunoreactivity for inhibin α and inhibin/activin (β_A and β_B) subunits was not observed in germ cells during the breeding season. No immunostaining was detected in control sections in which normal rabbit serum was substituted in place of the primary antibody (**F**). LC: Leydig cell; Se: Sertoli cell; Spg: spermatogonia; Spc: spermatocytes; Spd: spermatids. Scale bars (**C, D,E,F**) represent 40 μm.

the context of mature Sertoli cells and normal spermatogenesis *(20)*. Identification of the sites of expression and production of inhibin subunit messenger RNA and protein is critical to the understanding of the biology of inhibins *(20)*. In the present study, inhibin α and inhibin/activin (β_A and β_B) subunits were expressed in Leydig cells and Sertoli cells of black bears during the breeding season, showing that the Leydig cells and Sertoli cells may secrete dimetric and bioactive inhibins in the breeding season. Previous studies suggested that inhibin subunits may play some important roles in spermatogonial development *(21,22)* such as Sertoli cell proliferation *(23,24)*, and steroid biosynthesis *(24–27)*. Recent studies in black bears also demonstrated that 3β-HSD and P450arom were present in the same Leydig cells and Sertoli cells in the breeding season, indicating that Leydig cells and Sertoli cells of the black bears have the ability to

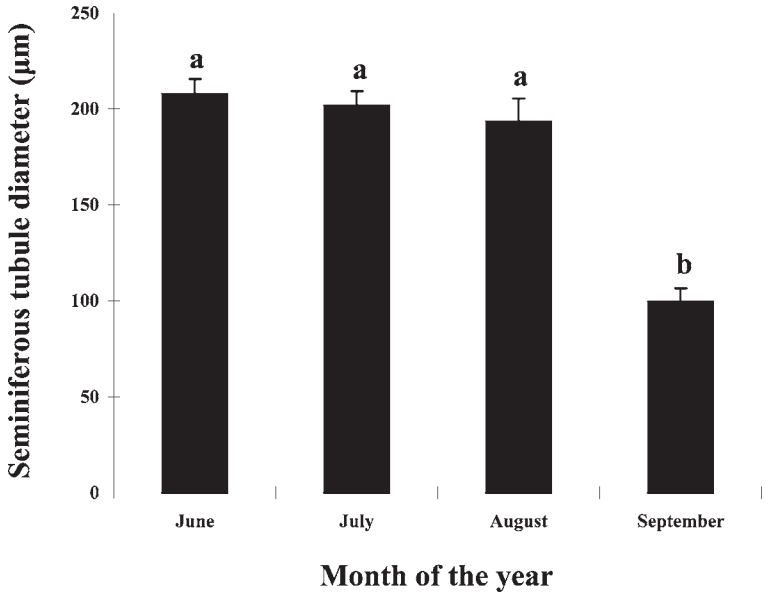

Fig. 2. Changes in seminiferous tubule diameters of wild male Japanese black bears as observed in June, July, August, and September. Values are expressed as the mean ± SEM. [ab]Values with different superscripts are significantly different. A value of $p < 0.05$ was considered significant.

secrete testosterone and estradiol *(13,17)*. These results concluded that the production of inhibin, and possibly activin, is dependent on the stage of the cycle of the seminiferous epithelium; these growth factors might, in cooperation with testosterone and estradiol, play a paracrine role in the differentiation of spermatogenic cells.

The survival of spermatogenic cells is dependent on gonadotropins as well as intratesticular testosterone induced by LH *(28,29)*. Testosterone is essential for the initiation and maintenance of spermatogenesis and regulating spermatogenesis at specific germ-cell transformation steps *(30)*. Previous studies in black bears showed that seasonal changes in spermatogenesis are correlated with changes in serum testosterone concentrations, and showed that all types of spermatogenic cells including mature spermatozoa were found in June *(13,17)*. In the present study, our results showed higher levels of blood inhibin, testosterone, and LH around June compared with other periods in August and September. Moreover, the entire spermatogenic cell population from spermatogonia to spermatozoa was present in June. These results may be explained by the fact that the resultant increase in LH pulse frequency stimulates a continued increase in testosterone secretions, which synergize with inhibin to become an effective negative feedback signal to control FSH secretion in the breeding season. Although these actions are influenced by the stage of the breeding season, and that inhibins are a direct product of the seminiferous tubules, its secretion is stimulated by the presence of advanced stages of spermatogenesis *(6)*.

As a classic hormone, it is well known that inhibins are stimulated by FSH and in turn regulate FSH production by negative feedback at the pituitary *(20)*. Administration of human recombinant inhibin to rats, castrated rams, and monkeys led to the specific suppression of FSH with no effect on testosterone or LH *(20)*. However, inhibins synergize with testosterone in the regulation of FSH, supporting the extensive data showing that testosterone can also suppress FSH *(20)*. Similar conclusions have been reached by studies in rams and primates exploring the inhibin hypothesis *(6,31)*. However, previous studies of some seasonal breeders, such as stallions *(5)* and golden hamsters *(32)*, suggested that testicular inhibin secretion may not be directly and immediately influenced by circulating FSH levels. In the present study, we tried to measure serum concentration of FSH by the heterologous radioimmunoassay. However, the assay did not succeed. In addition, there are no available assays for measuring inhibin A and inhibin B of bears. Therefore, further studies are needed to investigate the relationship among FSH, inhibin A, and inhibin B in Japanese black bears.

In conclusion, we have shown that the inhibin/activin α, βA, and βB subunits are colocalized in Leydig cells and Sertoli cells in the Japanese black bear testes during breeding season. In addition, seasonal changes in circulating inhibin showed positive correlations with those in testosterone and LH during the breeding season. These results suggest that the testes may secrete a dimetric inhibin involved in control of spermatogenesis, and that circulating inhibin may be a useful indicator of the testicular function in wild male Japanese black bears.

Materials and Methods

Animals

Thirty wild adult male Japanese black bears were captured in Gifu prefecture from the summer–autumn of 1998–

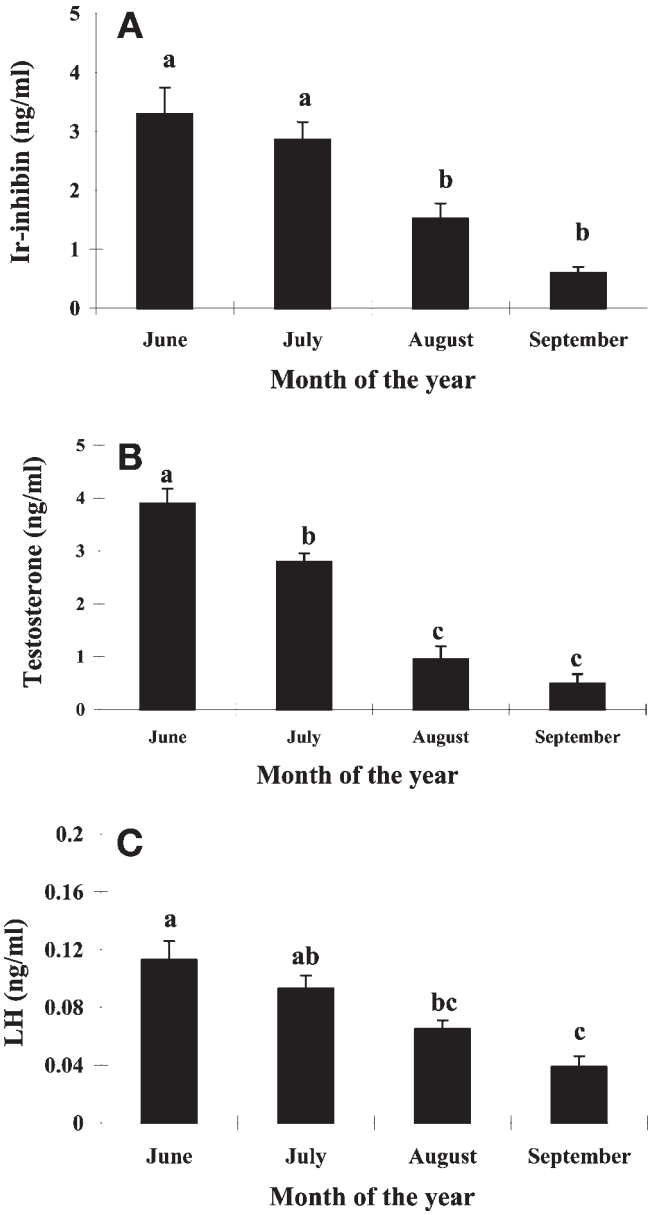

Fig. 3. Serum concentration of (**A**) immunoreactive (ir-)inhibin, (**B**) testosterone, and (**C**) LH of wild male Japanese black bears. Values are expressed as the mean ± SEM. [abc]Values with different superscripts are significantly different. A value of $p < 0.05$ was considered significant.

2002 with the permission of the former Environment Agency, Japan. On the next day after capture, black bears were immobilized by an intramuscular administration of Ketamine HCl [Veterinary Ketalar 50 (10 mg/kg)] and medetomidine HCl (Domitor 0.04 mg/kg). After immobilization, blood samples were obtained from the jugular vein into vacuum blood-drawing tubes and testes were surgically biopsied. The age of adult animals (≥3-yr-old) was judged according to the annual incremental lines in the tooth cementum of incisors. Testicular tissues obtained were immediately fixed for 13 h in Bouin's solution for histological and immunohistochemical observations.

Tissue Preparation

The testicular tissues obtained were dehydrated in a graded series of ethanol and embedded in paraffin wax. The paraffin-embedded tissues were serially sectioned at 4 μm thickness and mounted on glass slides coated with poly-L-lysine (Sigma Diagnostics. Inc., St. Louis, MO, USA).

Histology

Sections of testicular samples were stained with hematoxylin–eosin (HE) for observations of general histology. Ten seminiferous tubules per the black bear in June, July, August, and September were evaluated histologically using an Olympus photomicroscope with a ×40 objective lens. A movable cursor dot (approx 0.1 μm in diameter) was used for measuring the diameter of seminiferous tubules. Jandel Scientific Sigma Scan® Image Analysis software (Jandel Scientifics Corporation, Montgomeryville, PA) was used for processing measurements. The repeatability of measurements, expressed as a coefficient of variation for 10 measurements, was 0.74% at 10 μm and 5.8% at 0.7 μm.

Immunohistochemistry

Testicular sections were incubated with 10% normal goat serum to reduce background staining caused by the second antibody. The sections were then incubated with primary antibody (1:1000 or 1:2000) raised against porcine inhibin α chain (1-30)-NH2 conjugated to rabbit serum albumin, porcine inhibin/activin βA (81-113)-NH_2 (#305-24D) *(33)*, and cyclic acetyl human inhibin/activin βB (81-113)-NH_2 (#305-25D) *(33)* for 12 h at room temperature. The inhibin α subunit peptide was kindly provided by Dr. N. Ling, Neuroendocrine Inc, San Diego, CA, USA; the antibodies of inhibin/activin (βA and βB) were kindly provided by Dr. W. Vale, Salk Institute for Biological Studies, La Jolla, CA, USA. The sections were then incubated with a second antibody, goat anti-rabbit lgG conjugated with biotin and peroxidase with avidin, using a rabbit ExtrAvidin™ staining kit (Sigma, St. Louis, MO), followed by visualizing with 30 mg 3,3-diaminobenzidine (Wako, Tokyo, Japan) solution in 150 mL of 0.05 mol Tris-HCl l^{-1} buffer, pH 7.6, plus 30 μL H_2O_2. Finally, the reacted sections were counterstained with hematoxylin solution (Merck, Tokyo, Japan). The control sections were treated with normal rabbit serum (Sigma) instead of the primary antisera.

Radioimmunoassay (RIA) of Inhibin, LH, and Testosterone

Serum concentration of immunoreactive (ir-) inhibin was measured by double-antibody RIA as described previously *(34)* using bovine 32-kDa inhibin for radioiodination and antibovine antiserum (TNDH-1). The results were expressed in terms of 32-kDa bovine inhibin. The intra- and interassay coefficients of variation were 8.0% and 16.2%. Serum concentrations of LH were measured using the heterologous double-antibody canine RIA methods as described

previously *(35)*. Iodinated preparations were rat LH-I-5 and the antisera were anti-ovine LH (YM #18). Results were expressed as canine LH (LER-1685-1). Intra- and interassay coefficients of variation were 12.5% and 15.1%.

Serum concentrations of testosterone were determined by double-antibody RIA systems using ^{125}I-labeled radioligands as described previously *(36)*. Antiserum against testosterone (GDN 250) was kindly provided by Dr. G.D. Niswender (Animal Production and Biotechnology, Colorado State University, Fort Collins, CO). The intra- and interassay coefficients of variation were 6.3% and 7.2%.

Statistical Analyses

Mean values (± SEM) were calculated and analyzed using two-way ANOVA. Duncan's multiple-range test was used for detection of significant differences using the SAS computer package *(37)*. A value of $p < 0.05$ was considered significant.

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