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Changes in plasma gonadotropins, inhibin and testosterone concentrations and testicular gonadotropin receptor mRNA expression during testicular active, regressive and recrudescence phase in the captive Japanese black bear (*Ursus thibetanus japonicus*)

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Abstract

Male Japanese black bears (*Ursus thibetanus japonicus*) have an explicit reproductive cycle. The objective of this study was to clarify the variation of plasma testosterone, FSH, inhibin, LH levels and testicular gonadotropin receptor mRNA expression of male bears associated with their testicular activity. Notably, this study investigated peripheral FSH concentration and localization of gonadotropin receptor mRNAs for the first time in male bears. Blood and testicular tissue samples were taken from captive, mature, male Japanese black bears during testicular active, regressive and recrudescence phases. Plasma hormone concentrations were measured by immunoassays, and gonadotropin receptor mRNA expression in the testis was investigated by *in situ* hybridization technique and also by real-time PCR. There were significant variations in plasma testosterone and inhibin concentrations. Changes in FSH concentration preceded these hormones with a similar tendency. Hormones started to increase during denning, and achieved the highest values at the end of the recrudescence phase for FSH and in the active phase for testosterone and inhibin. These changes in hormone concentrations were accompanied by testicular growth.

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***In situ* hybridization analysis revealed that FSH and LH receptor mRNA was possibly expressed in Sertoli cells and Leydig cells, respectively, as they are in other mammals. However, neither plasma LH concentration nor testicular gonadotropin receptor mRNA expression level varied significantly among the sampling months. These results suggest that FSH, inhibin and testosterone have roles in testicular activity in male bears. This study provides important endocrine information for comprehending seasonal reproductivity in male Japanese black bears.**

Key words: follicle-stimulating hormone, gonadotropin receptor, inhibin, Japanese black bear, luteinizing hormone

Introduction

The Japanese black bear (*Ursus thibetanus japonicus*), a subspecies of the Asiatic black bear living in Japan, is a long day seasonal breeder. In this species, both males and females are known to have a pronounced seasonality in their gonadal activity with the breeding season from June to July. In males, active spermatogenesis and testicular steroidogenesis are recognized during the pre-breeding to breeding season¹²⁾, and blood testosterone concentrations decrease and the testes undergo regression when breeding season is over¹¹⁾. The onset of spermatogenesis occurs during the denning period. Komatsu *et al.*¹¹⁾ reported that testicular recrudescence in male Japanese black bears was observed around March, which is in the latter half of denning.

Major endogenous factors that transfer the environmental information and mediate seasonal changes of gonadal function are the secretion of gonadotropins, namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary gland¹³⁾. Not only blood gonadotropin concentrations but also many other factors, such as negative feedback of inhibin or sex steroids, and gonadotropin receptor expression, are involved in controlling reproductive seasonality. However, the behavior of these factors during denning is less known in male bears, compared to many reports on sex steroid concentrations^{11,19,26,31)}. According to our knowledge, the annual changes in FSH and

inhibin concentrations have not been reported yet in male bears, except for inhibin concentration around the mating season³³⁾. Thus, the reproductive endocrinology of male bears during testicular recrudescence has yet to be elucidated. In the present study, we investigated the changes of plasma concentration of gonadotropins, inhibin and testosterone, and testicular gonadotropin receptor mRNA expression during testicular recrudescence using captive, male Japanese black bears.

Materials and Methods

Animals and sampling procedure: Samples were obtained from sexually mature, male Japanese black bears, between the ages of 5 and 15, kept at Ani-mataginosato Bear Park located in Akita prefecture, northeastern Japan (N 40°, E 140.1°). In this park, male and female bears were kept separately in outdoor runs throughout the non-denning period (from May to November). In early December, bears were moved to indoor rooms for denning. During the denning period (from December to the following April), they were not provided any food but had free access to water.

Blood and testicular tissue sampling was performed in listed months in Table 1. The months were categorized as follows: testicular active phase (May, June and July), regressive phase (October and November) and recrudescence

Table 1. The number of the bears used for sampling for each measurement or detection.

Year		2007				2008				2009			
Month		Jun*	Oct**	Nov**	Dec	Jan	Feb	Mar	Apr	Jul*	Nov**	May*	Jun*
Subject	Assay												
Testosterone	Enzyme immunoassay	3		4	3	3	3	3	3	4			
FSH	Radioimmunoassay	3	4	4	3	3	3	3	3	4			
Inhibin	Radioimmunoassay	3	4	4	3	3	3	3	3				
LH	Radioimmunoassay	3	4	4	3	3	3	3	3	4			
LH pulse	Radioimmunoassay										3	4	
Testicular size				4		3	3	3	3	4			
Receptor expression	<i>in situ</i> hybridization			4		3	3	3	3	4			
	Real time PCR										6		5

The identical bears were used for plasma, testicular size and/or tissue sampling in each month. In addition, the same three bears were used through the denning period, December 2007 to April 2008. Sampling period was categorized as testicular active* (May to July), regressive** (October and November) and recrudescence (December to April) phase.

phase (December to the following April). During the non-denning period, bears were randomly chosen for sampling from thirty mature male bears kept in this park. On the other hand, samples were continuously taken from same 3 bears throughout the denning period.

For sampling, the bears were anesthetized by blow dart injections with combinations of the following drugs: 3.0 mg/kg zolazepam HCl and tiletamine HCl cocktail (Zoletil®, Virbac, Carros, France) and 40 µg/kg medetomidine HCl (Domitor®, Meiji Seika, Japan). All samplings were performed with the supervision and approval of the Animal Care and Use Committee of Hokkaido University. Sampling for plasma, testicular size and tissue were performed as follows.

Blood samples were taken from the jugular vein of bears for FSH, inhibin, LH and testosterone assays. Samples were transferred to a heparin tube, then plasma was collected by centrifugation at 3,000 rpm for 10 min and stored at -30°C until hormone measurement. The left testicular size was measured over the scrotum with calipers and calculated by the following formula²⁵: $(\text{length} \times \text{width} \times \text{depth})^{1/3}$. Then, testicular tissues were obtained by biopsy. For *in situ* hybridization, one piece of testicular tissue was embedded in OCT compound (Sakura

Finetechnical Co., Ltd., Tokyo, Japan) and immediately frozen with dry ice. Blocks were stored at -30°C until experiment. For real-time quantitative PCR assay, testicular tissue was put in cryotubes (Nunc, Roskilde, Denmark), frozen with dry ice, and stored at -80°C until RNA extraction. Meloxicam (Metacam®, Boehringer Ingelheim, Germany) was injected subcutaneously at a dosage of 0.2 mg/kg for analgesia immediately after the biopsy.

We performed experiments to detect the LH pulsatile secretion with 3 bears in November, 2008 and 4 bears in May, 2009 by taking plasma samples for 4 hr duration. After immobilization, the bears were administered a continuous drip infusion of Ringer's acetate solution (Solulact F®, Terumo, Japan) containing 1 mg/ml Zoletil (zolazepam HCl and tiletamine HCl cocktail) with an infusion rate 1.0 ml/kg/hr for extended anesthesia through an intravenous line in one jugular vein. The blood samples were collected from the other jugular vein every 5 min for 4 hr (49 samples/bear) in the heparin tube. Then, plasma was separated and stored as described above. After blood sampling for 4 hr duration, testicular sampling from these bears was performed in November, 2008.

Radioimmunoassay: Plasma FSH, inhibin

and LH concentrations were measured at Tokyo University of Agriculture and Technology by heterologous double-antibody radioimmunoassay^{3,22)}. The chloramine-T method was used for hormone iodination as previously described²⁹⁾.

Plasma FSH concentrations were measured using ¹²⁵I-labeled rat FSH (NIDDK-I-5) and anti-human FSH rabbit serum (M91) as the primary antibody. Intra-assay variation was 8.2% for FSH measurement. Plasma inhibin concentrations were measured using a rabbit antiserum against purified bovine inhibin (TNDH-1) and ¹²⁵I-labeled 32-kDa bovine inhibin. Intra-assay variation was 6.5% for inhibin measurement. Plasma LH concentrations were measured with ¹²⁵I-labeled rat LH (NIDDK-I-5) and anti-ovine LH rabbit serum (YM #18; kindly provided by Dr. Y. Mori, University of Tokyo, Tokyo, Japan). Intra-assay variation was 6.8% for LH measurement. The results for FSH, inhibin and LH were expressed in terms of purified canine FSH (LER-1685-1), 32-kDa bovine inhibin and canine LH (LER-1685-3A), respectively.

Enzyme immunoassay: Testosterone was extracted for plasma samples as follows: 100 µl of the plasma sample was placed in a glass tube after vortexing. Then, 2 ml of diethyl ether was added, and the tubes were capped with a Teflon-sealed plug and shaken vigorously for 10 min. Subsequently, the samples were left to rest for 10 min. The ether layer was recovered by decanting to another tube after snap-freezing at -80°C, and then by promoting evaporation in a 42°C bath. The residue was redissolved in 200 µl of assay buffer (sodium phosphate [pH 7.4], with 8.7 g/l sodium chloride and 1 g/l BSA) by vortex mixing for 10 min. As a result, samples were diluted 2-fold for testosterone analysis. The extracts were frozen at -20°C until the assay was performed.

Testosterone levels were measured using an enzyme immunoassay (EIA). Testosterone-3-

CMO-HRP (FKA101) was obtained from Cosmo Bio (Tokyo, Japan). Standard testosterone (Cayman, Ann Arbor, MI, USA) was diluted in the assay buffer. Anti-testosterone serum (first antibody, FKA102-E; Cosmo Bio) was diluted 1,200,000-fold with assay buffer. Anti-rabbit γ-globulin serum (Seikagaku Co., Tokyo, Japan) was used for the secondary antibody. The minimum detectable level of testosterone was 4.9 pg/well, and the intra- and inter-assay coefficients of variation were 9.5% and 17.3%, respectively.

In situ hybridization: The procedure of *in situ* hybridization used in this study has been described by Nio *et al.*²⁴⁾. Briefly, three non-overlapping antisense oligonucleotide probes (45 mer in length: Table 2), complementary to the nucleotide residue of the polar bear (*Ursus maritimus*) gonadotropin receptor mRNA sequence (GenBank Accession number: AF69791 for FSH receptor and AF69790 for LH receptor) were prepared by Hokkaido System Science Co., Sapporo, Japan. The oligonucleotides were labeled with ³³P-dATP using terminal deoxyribonucleotidyl transferase. The sections were fixed with 4% paraformaldehyde for 15 min and acetylated for 20 min with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0). Hybridization was performed at 42°C for 10 hr adding 10,000 cpm/µl ³³P-labeled oligonucleotide probes. The sections were rinsed once at room temperature for 30 min in 2 × SSC (1 × SSC: 150 mM sodium chloride and 15 mM sodium citrate) containing 0.1% sarkosyl and twice at 55°C for 40 min 0.1 × SSC containing 0.1% sarkosyl, and dehydrated in series of ethanol. Then, they were either exposed to Biomax MR film (Kodak, Rochester, NY, USA) for 2 weeks or dipped in autoradiographic emulsion (NTB-2; Kodak), exposed for approximately 10 weeks at 4°C. The sections for autoradiography were counterstained with hematoxylin after development. The specificity of the hybridization was confirmed by the

Table 2. Sequence of probes (45 mer) for *in situ* hybridization technique and primers for real time PCR analysis, designed by *Ursus maritimus* FSH receptor (Genbank Accession number; AF169791) and LH receptor (AF169790).

Probe		Sequence (5'→ 3')		
FSH receptor-1		GGACAGGTTGGAGAACACATTTGCCTCGATCGCTTCCAAGACATC		
FSH receptor-2		GGCTGGCCTCCATGAGTGCACAAATGTATCCAGACTGGGAAGCT		
FSH receptor-3		TGGATTAAATGCATCTGGCTTAGGGGAGCAAGTCACGTCAACCAC		
LH receptor-1		TGCTCCAGGCTCGATGTACACCAGGTTTTTGGTGTCTGGATCAG		
LH receptor-2		CAATGAGTGTCTGGATGGACTCCAGCCCATAGCTAGGCAAGGCTT		
LH receptor-3		CCCGGAAGGCTCCATTGTGCATCTTCTCCAGGTGGGCATTTTCCT		
Primer		Sequence (5'→ 3')	Length	Product size
FSH receptor	forward	TGTGTTCTCCAACCTGTCCA	20	146
	reverse	CAGCTGGCAAATGCTGAATA	20	
LH receptor	forward	CACCTGGAGAAGATGCACAA	20	135
	reverse	GGATGATGTGGCAATGAGTG	20	
HPRT	forward	GAAACAGTGACGGGTCCATT	20	89
	reverse	GACTTTGATGTCCCCTGTTGA	21	
β -actin	forward	CTCTTCCAGCCTTCCTTCCT	20	172
	reverse	TGATCTCCTTCTGCATCCTGT	21	

disappearance of the signals with an excess dose of unlabeled antisense probes.

Quantitative real-time PCR analysis: The relative FSH receptor (FSHr) and LH receptor (LHr) mRNA expression were analyzed using the $\Delta\Delta C_t$ method¹⁵⁾. Total RNAs were extracted from each testicular sample and treated with DNase I using an RNAquaous-4PCR Kit (Ambion, Austin, TX, USA) following the manufacturer's instructions. Then cDNA was synthesized from the amount of 1 or 2 μ g total RNA with random hexamer using a SuperscriptTM III first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Each receptor cDNA was amplified with primers designed using Primer 3 software with the above-cited polar bear (*Ursus maritimus*) gonadotropin receptor mRNA sequence. Primers for hypoxanthine guanine phosphoribosyl transferase (HPRT) gene and β -actin gene were also designed and used as the internal control. Primer sequences and length of the product for each gene is listed in Table 2. PCR reactions

were conducted using 1 μ l aliquots of the cDNA samples (diluted to contain 10 ng total RNA), subjected to amplification by ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster city, CA, USA) in 19 μ l mixture in the presence of 10 μ l *Power SYBR[®] Green* PCR Master Mix (Applied Biosystems), 8 μ l dH₂O and 0.5 μ l each of 100 μ M primers. PCR reactions consisted of an initial denaturing cycle at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of amplification defined by denaturation at 95°C for 15 sec, annealing and extension at 60°C for 1 min, followed by post-amplification dissociation curve analysis. Each specimen was run in duplicate. Obtained data were analyzed by Sequence Detection Software (SDS v1.7) for determining C_t (Threshold cycle) values of each sample for quantifying the start templates in the reaction. To obtain standardized quantitative results, the amount of FSHr and LHr mRNA expression was normalized to HPRT or β -actin (ΔC_t value: C_t [target gene] – C_t [Control gene]). Then, the relative abundance of mRNA in June to November was calculated by $\Delta\Delta C_t$

(ΔCt [Jun] $-\Delta\text{Ct}$ [Nov]). The ratio of mRNA concentration between active and regressive phases was expressed as $2^{-\Delta\Delta\text{Ct}}$.

Statistical analysis: The significance of differences among months was statistically analyzed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Plasma concentrations of testosterone, FSH, inhibin and LH, and testicular size were assessed using one-way analysis of variance (ANOVA), followed by the Tukey test for *post hoc* testing. In the real-time PCR assay, Student *t* test was performed to determine the significance. Comparisons were considered significant at $P < 0.05$. All values are represented as mean $\pm$ SEM.

Results

Changes in plasma concentration of gonadotropins, inhibin and testosterone and testicular size

Variations in plasma testosterone, FSH, inhibin and LH concentrations are shown in Fig. 1. Significant seasonal variations were found in plasma testosterone and inhibin concentrations ($P < 0.05$) (Fig. 1A and 1C). The concentrations of these hormones were lowest in January (testosterone: 0.05 ± 0.01 ng/ml) and December (inhibin: 1.03 ± 0.19 ng/ml), respectively, and gradually increased through the recrudescence phase, then reached the highest concentrations in the active phase (3.63 ± 0.81 and 3.41 ± 0.76 ng/ml, respectively). Differences between the regressive and active phases, and also between the early recrudescence phase (December to February) and the active phase were significant ($P < 0.05$). Similarly, plasma FSH concentration was comparatively low in the regressive phase (2.91 ± 1.13 ng/ml), then increased gradually in the recrudescence phase (Fig. 1B), achieving the highest value in April (24.32 ± 13.09 ng/ml). Though the difference was not statistically significant when analyzed with

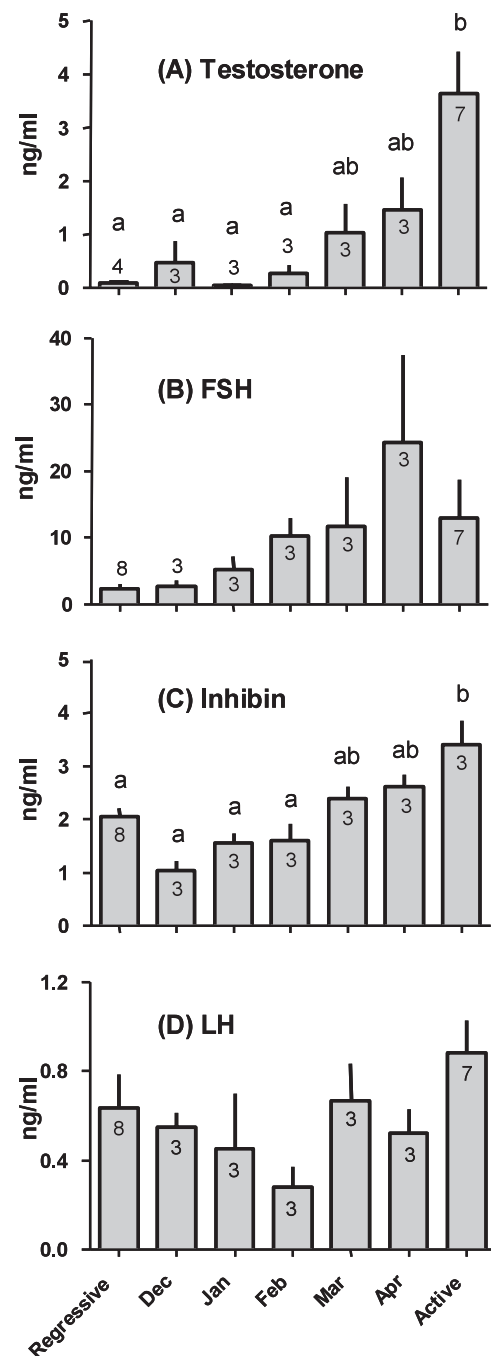


Fig. 1. Change in plasma concentrations of testosterone (A), FSH (B), inhibin (C) and LH (D) in male Japanese black bears during testicular active, regressive and recrudescence phases. Data of the recrudescence phase are represented monthly. Testosterone, FSH and inhibin concentrations tend to increase during denning, when the same 3 bears were used, and reach a maximum in April (FSH) or in the active phase (testosterone and inhibin). Different small letters indicate significant difference ($P < 0.05$). Error bars mean SEM. The number of plasma samples used in each month is shown in the figure.

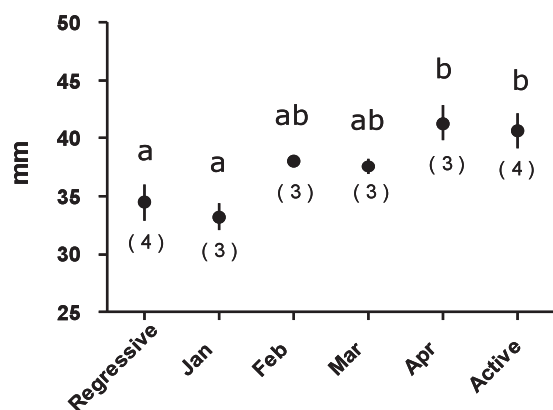


Fig. 2. Variations in the size of left testis of bears calculated by the following formula: $(\text{length} \times \text{width} \times \text{depth})^{1/3}$. The same 3 bears were utilized during the recrudescence phase. Testicular size shows significant increase. Different letters indicate significant difference ($P < 0.05$) and error bars mean SEM. The number of plasma samples used in each month is shown in the figure.

all investigated months ($P = 0.131$), a tendency could be confirmed ($P = 0.086$: repeated one-way ANOVA) when focusing just on the recrudescence phase. Because plasma LH concentration did not vary significantly (Fig. 1D; $P > 0.05$), experiments to detect LH pulse for 4 hr duration were performed. However, evidence of pulsatile secretion of LH was not recognized (data not shown).

Seasonal variations in left testicular size are shown in Fig. 2. The testes became larger during the recrudescence phase. The size in the regressive phase (34.5 ± 2.6 mm) and January (33.2 ± 2.1 mm) was significantly smaller than in the late recrudescence (April; 41.3 ± 2.6 mm) and active phases (40.7 ± 3.1 mm) ($P < 0.05$).

Expression of gonadotropin receptor mRNA

By *in situ* hybridization, signals for FSHr mRNA were observed inside the seminiferous tubules along the basement membrane (Fig. 3A–B). On the other hand, signals for LHr mRNA were seen in the interstitium (Fig. 3E–F) during the active phase. Identical results were obtained by using three non-overlapping probes. Meanwhile, cocktails of two probes (FSH receptor-1 and FSH receptor-2: Table 2) were

utilized for FSHr mRNA detection, in order to strengthen the visibility of the image, since the signals were weak in the preliminary experiment using each probe separately. The testes of the regressive and recrudescence phases also expressed FSHr and LHr mRNA, without apparent changes in expression sites (Fig. 3) or signal intensities (Fig. 4). To perform further quantitative analysis, we compared the receptor mRNA expression level by real-time PCR. The result revealed no significant change between the active and regressive phases (Fig. 5; $P > 0.05$). However, FSHr gene expression was comparatively low in the active phase, about 0.74-fold, to that in the regressive phase (Fig. 5A; $P = 0.068$). The identical results were obtained using two control genes, HPRT and β -actin.

Discussion

This study demonstrates distinct variations in plasma testosterone concentrations with the highest levels in the active phase, and a similar near-significant change in plasma FSH which precedes the change in testosterone. The change in plasma testosterone revealed in this study corresponded well with previous data^{11,25,31,34}. It is well known that the change in testosterone concentration is positively correlated with the change in spermatogenesis in bears^{11,25,31,34}. Plasma testosterone concentration is directly related to testicular activity because intratesticular testosterone is crucial for the maintenance of advanced spermatogenic cells³⁵. On the other hand, FSH has a key role in reinitiating spermatogenesis in seasonal breeders through regulating the spermatogonial population^{16,20}. In the present study, we described peripheral FSH concentration in males for the first time in any bear species. Plasma FSH concentration tended to increase preceding the testosterone increase, and achieve the highest levels before the breeding season. These

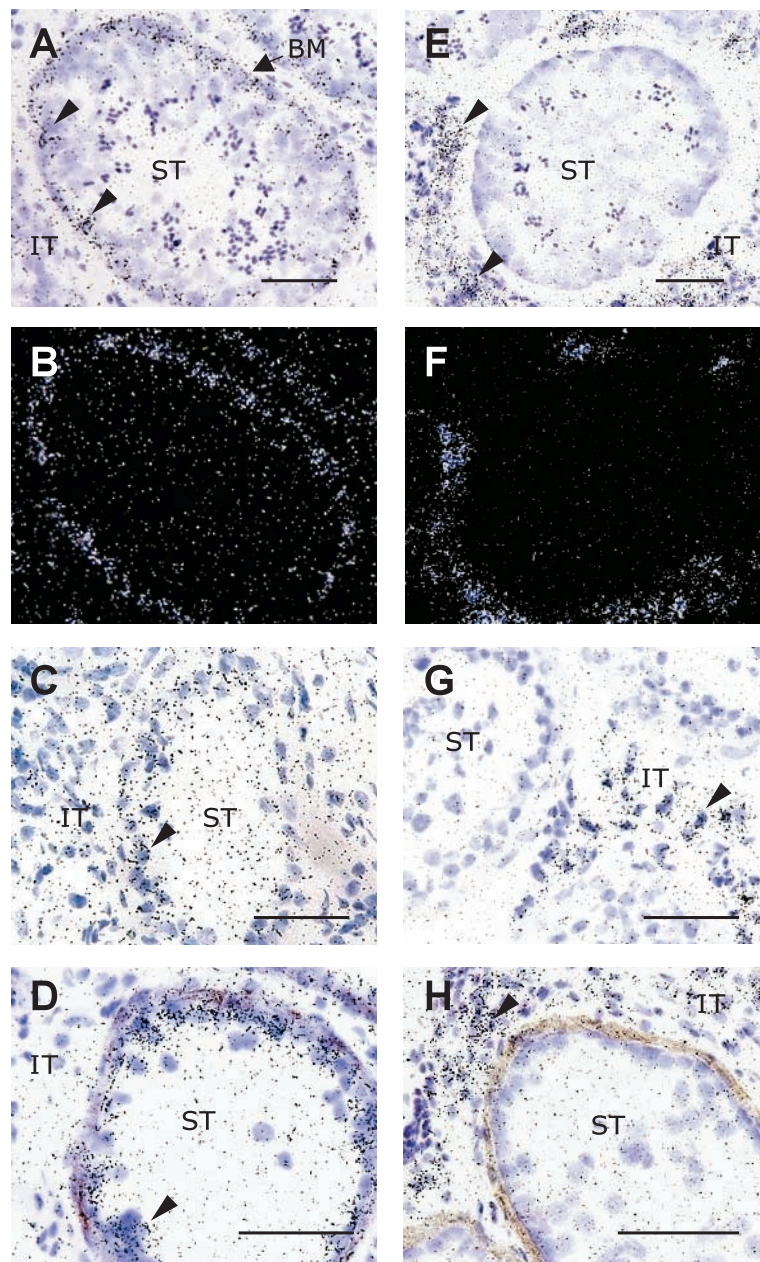


Fig. 3. Microscopic images of sections from bear testes processed by *in situ* hybridization technique. Signals of mRNA are shown as silver grains in a bright-field image (arrow heads). FSHr mRNA expressions are found inside the seminiferous tubules (ST) along the basement membrane (BM) in the active phase (A: bright-field, B: dark-field), the regressive phase in C) November and recrudescence phase in D) March. On the other hand, LHr mRNA is expressed in the interstitial tissue (IT) in the active phase (E: bright-field, F: dark-field), the regressive phase in G) November and recrudescence phase in H) March. Figs. 3B and 3F are identical fields to Figs. 3A and 3E. Bars represent 50 μ m.

characteristics were in agreement with reports that showed the maximum FSH concentrations before the breeding season in blue foxes²¹⁾, rams¹⁴⁾, ground squirrels¹⁾ and roe deer³⁰⁾. In the present study, the increase in plasma FSH

concentration was also accompanied by an increase in testicular size. These findings indicate that FSH in the Japanese black bear may have some role in reinitiating testicular growth as it does in other mammals.

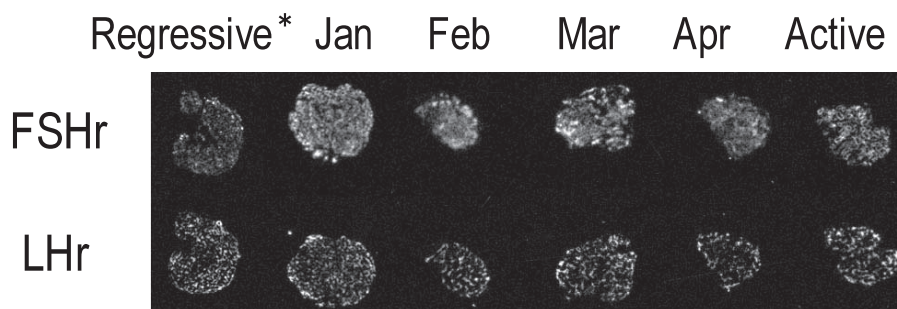


Fig. 4. X-ray images showing mRNA expressions for FSHr and LHr in testicular biopsy samples detected by *in situ* hybridization technique. Messenger RNA expressions are seen as white colored. The signals for both receptors are found in bear testis in all investigated months. The experiment for FSHr detection in the regressive phase (*) was performed with only one probe (FSH receptor-1) so that expression level is not able to be compared with other phases using cocktail of two probes. The sections during the recrudescence phase in this figure were taken from the same bear. Bars represent 1 mm.

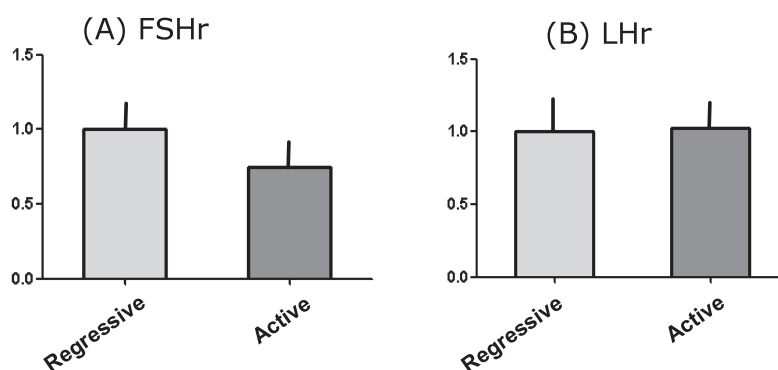


Fig. 5. Relative concentrations of FSHr (A) and LHr (B) mRNA in testicular tissue between regressive and active phases. Receptor mRNA abundance was corrected by HPRT mRNA and represented the value in the regressive phase as a criterion ($\Delta\Delta C_t$ method). No significant difference is detected in relative amount of mRNA for both receptors between two phases ($P > 0.05$).

Plasma inhibin concentration also showed a significant change with the maximum value in the active phase and the minimum value in December. Weng *et al.*³³⁾ reported higher serum inhibin in June and July than in August and September in wild male Japanese black bears. Therefore, it appears that there is a significant annual change in its concentration. The change in plasma inhibin, which synchronized with plasma testosterone, indicates that plasma inhibin changes in correspondence with the testicular activity. It is thought that inhibin is secreted from testicular somatic cells in Japanese black bears because immunoreactive-inhibin subunits were detected in both Sertoli and Leydig cells³³⁾. In some mammalian species, the major site of inhibin production in the testis

is Sertoli cells, and inhibin secreted from Sertoli cells acts as a negative feedback on FSH secretion²⁾. Since the change patterns in FSH and inhibin concentrations in this study were similar to previous reports showing that changes in FSH concentration preceded changes in inhibin concentration in rams and hamsters^{8,28)}, inhibin in bears may also have a negative feedback role on FSH secretion.

In contrast to FSH, inhibin and testosterone, plasma LH concentration did not change when measured by LH radioimmunoassay, a method which has been successfully adopted for various mammals, such as dogs²³⁾, cattle¹⁸⁾, monkeys¹⁷⁾ and horses²²⁾. To date, peripheral LH concentration of males have been reported in wild polar bears⁷⁾, wild Japanese black bears³³⁾

and captive American black bears⁴⁾, leading to controversial results in which significant differences were seen in the former two, which focused only on the mating to post-mating season, but not in the latter performing seasonal sampling. It is thought that change in LH concentration is comparatively clear during the mating to post-mating season, but unclear during the remainder of the year, since the results of the present study were similar to the report in American black bear⁴⁾. However, there could be an alternative reason explained by a pattern of LH secretion. It is well known that LH is released in a pulsatile manner. We failed to find LH pulses in the present study, but plasma LH levels were not constant throughout 4 hr sampling. This fluctuation may affect the statistical analysis of the results, especially with our small sample size, and make it difficult to obtain unified results among male bears.

The results of the *in situ* hybridization revealed a differential expression of FSHr and LHr expressed in the seminiferous tubules and the interstitium, respectively. These findings suggest that, possibly, Sertoli cells express FSHr and Leydig cells express LHr in bear testis as in other mammals^{10,27,32)}. Changes in the number of gonadotropin receptors are known to be corresponding factors in the reproductive cycles in some seasonal breeders^{9,28)}. However, no apparent difference was seen in mRNA expression of either FSH or LH receptors in the bear testis by *in situ* hybridization and by quantitative real-time PCR, despite one-quarter lower expression in FSHr mRNA in the active phase. This decrease in FSHr mRNA might support the previous report on the American black bear⁵⁾, suggesting that FSHr mRNA was expressed more abundantly in January (recrudescent phase) than in May (active phase). FSHr expression may increase as early as in the regressive phase and remain at a high level until the recrudescence of spermatogenesis. On the other hand, LHr mRNA expression was reported to be higher in May than in January⁶⁾.

The reason for the inconsistency between previous findings and this study is unclear. The onset of denning might have some negative influence on the LHr mRNA expression. However, the expression of these two receptors throughout the recrudescence phase indicates that gonadotropin could act through those receptors at the onset of spermatogenesis and steroidogenesis, which occur during hibernation as shown in a previous study¹¹⁾.

In summary, we demonstrated the plasma concentration of gonadotropins, testosterone and inhibin and gonadotropin receptors mRNA expression in captive Japanese black bears in the present study. A near-significant change in plasma FSH concentration preceded the significant parallel changes in plasma testosterone and inhibin concentrations. Changes in plasma concentration of these hormones were accompanied by testicular growth. FSH and LH possibly target Sertoli cells and Leydig cells, respectively, and receptors were expressed throughout the testicular active, regressive and recrudescence phases. These findings indicate that testosterone, FSH and inhibin have roles in testicular activity in male bears during testicular recrudescence. This report is the first examination of peripheral FSH concentration and localization of gonadotropin receptor mRNA in male bears, and this endocrine information should be useful for further investigation for their reproductive seasonality.

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