Journal of Reproduction and Development, Vol. 64, No 5, 2018

—Original Article—

Relationships of plasma insulin-like peptide 3, testosterone, inhibin, and insulin-like growth factor-I concentrations with scrotal circumference and testicular weight in Japanese Black beef bull calves

Mitsuhiro SAKASE[2)], Keita KITAGAWA[1)], Masahiko KIBUSHI[1, 3)], Noritoshi KAWATE[1)], W. W. P. N. WEERAKOON[1)], M. A. HANNAN[1)], Namiko KOHAMA[2)] and Hiromichi TAMADA[1)]

[1)]*Department of Advanced Pathobiology, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka 598-8531, Japan*

[2)]*Hokubu Agricultural Institute, Hyogo Prefectural Technology Center for Agriculture, Forestry and Fisheries, Hyogo 669-5254, Japan*

[3)]*Asago Animal Hygiene Institute of Hyogo Prefecture, Hyogo 669-5243, Japan*

Abstract. This study was conducted to clarify the relationships of plasma concentrations of insulin-like peptide 3 (INSL3), testosterone, inhibin, and insulin-like growth factor-I (IGF-I) with scrotal circumference and testicular weight in Japanese Black beef bull calves (n = 20), from birth to pre-puberty. Monthly blood sampling (0 to 7 months) and scrotal circumference measurements (0 to 7 months) were performed. Testicular weight was recorded immediately after castration at 7 months. Plasma INSL3, testosterone, inhibin, and IGF-I concentrations were measured either by enzyme immunoassay or time-resolved fluorescence immunoassay. The correlation coefficients of these hormonal concentrations with scrotal circumference were significant ($P < 0.0001$) and it was higher for INSL3 ($r = 0.647$) than for testosterone ($r = 0.597$), IGF-I ($r = 0.400$), and inhibin ($r = –0.453$). Calves with heavier testes (> 60 g) at castration (7 months) had higher ($P < 0.05$) plasma INSL3 (from 3 to 7 months) and inhibin (from 1 to 4 months) concentrations than those with lighter testes (< 60 g). The calves with heavier testes at castration had larger ($P < 0.05$) scrotal circumference than those with lighter testes from 3 to 7 months. In conclusion, blood INSL3 concentrations may be the best functional indicator among the hormones analyzed for determining total testicular volume during pre-puberty in bull calves. In addition, inhibin and INSL3 concentrations in early calfhood may be functional predictors for testicular weight at pre-puberty.

Key words: Bull calf, Inhibin, Insulin-like growth factor-I (IGF-I), Insulin-like peptide 3 (INSL3), Testicular size

(**J. Reprod. Dev. 64:** 401–407, 2018)

Insulin-like peptide 3 (INSL3) is secreted from Leydig cells in the testis in male mammals [1]. INSL3 is a stimulatory factor for testicular descent during fetal development in mice [2, 3] and for spermatogenesis during and after puberty in rats [4] and pigs [5]. Changes in INSL3 concentrations in peripheral blood during growth of male mammals have been reported in men [6–8], male rats [9, 10], bulls [11], dogs [12], and goats [13]. Furthermore, plasma INSL3 concentrations in beef bulls were shown to increase during neonatal and pubertal periods and the changes during puberty were different from those of testosterone concentrations [11].

In bulls, scrotal circumference increases gradually before 25 weeks of age, grows rapidly during the peripubertal phase, and then increases gradually again at maturity [14]. The scrotal circumference has been reported as a reliable predictor of puberty, and bulls with a smaller scrotal circumference were more likely to have unsatisfactory semen quality [14]. Positive correlations between blood INSL3 concentrations and testicular size during pubertal development have been previously reported in men [8] and goats [13]. However, the association between testicular size and peripheral levels of INSL3 during development are yet to be elucidated in bulls.

Inhibin is secreted mainly from Sertoli cells in testes and inhibits follicle-stimulating hormone (FSH) secretion from the pituitary gland [15, 16]. Serum inhibin concentration declines in bulls from birth to 9 months of age [17]; however, there have been no reports elucidating the relationship between blood inhibin concentrations and testicular size in developing bulls.

It has been suggested that a high plane of nutrition during calfhood positively affects scrotal circumference size and the onset of puberty in beef bulls [18, 19]. These studies showed increased plasma insulin-like growth factor I (IGF-I) concentrations during puberty in bulls with high-plane nutrition than those with low-plane nutrition [18, 19], suggesting that levels of IGF-I in peripheral blood may be associated with testicular development.

This study was conducted to clarify the relationships of plasma INSL3, testosterone, inhibin, and IGF-I concentrations with scrotal circumference and testicular weight in Japanese Black beef bull calves from birth to pre-puberty.

Received: March 14, 2018
Accepted: June 16, 2018
Published online in J-STAGE: July 6, 2018

Correspondence: N Kawate (e-mail: nkawate@vet.osakafu-u.ac.jp)

Materials and Methods

Animals

Japanese Black beef bull calves (n = 20) raised in an experimental beef cattle station at the Hokubu Agricultural Institute, Hyogo Prefectural Technology Center for Agriculture, Forestry and Fisheries in Japan were used for the present study. These bulls remained normal in appearance and healthy during all experiments. The animals were kept under natural light in an open shelter covered by a roof and remained with their dams until weaning at 5 months of age. The bull calves were fed calf starter pellets (TDN, 87.5%; CP, 23.9%; DM basis) from 7 days after birth, and the amounts of calf starter were gradually increased until 2.5 months of age. From 2.5 to 7 months of age, the calves were fed concentrate (TDN, 77.8%; CP, 17.7%; DM basis) to meet or exceed Japanese Feeding Standard recommendations for beef calves. Additionally, the calves were fed timothy hay *ad libitum* from 7 days after birth. Body weight was recorded monthly from 0 to 7 months of age. The scrotal circumference of the bulls was recorded monthly from 1 to 7 months of age, using a flexible tape measure, around the greatest diameter of the scrotal sac. The experiments were approved by the Hokubu Agricultural Institute, Hyogo Prefectural Technology Center for Agriculture, Forestry and Fisheries. The procedures used in the animal experiments complied with the guidelines for the Proper Conduct of Animal Experiments in Academic Research Institutions in Japan.

Blood sampling

Blood samples were obtained monthly from the calves from 0 to 7 months of age. They were collected from the jugular vein into heparinized tubes and immediately placed on ice. The blood was centrifuged at 1700 × *g* for 15 min at 4°C and the separated plasma was then stored (−30°C) until the assay.

Castration

The bull calves were surgically castrated at 7 months of age. The calves were sedated with xylazine hydrochloride (Celactal 2% Injection solution, Bayer, Tokyo; 0.1 mg/kg IV) 10 to 20 min before surgery. The calves were held in the lateral recumbent position and the surgical area was cleaned with a disinfectant solution (benzalkonium chloride; Osban, Nihon Pharmaceutical, Tokyo, Japan) and sterilized with a povidone iodine spray solution (Isodine Animal 10%, DS Pharma Animal Health, Osaka, Japan). Incisions were made vertically on the skin of the anterior scrotum and testicular tunica to expose the testes. Each testicle was pushed through the opening and exteriorized. The exposed spermatic cord was ligated with a surgical silken suture and severed with a sterile blade approximately 1 cm distal to the ligation. The calves were treated daily for 7 days after surgery with a mixture of dihydrostreptomycin sulfate and benzylpenicillin procaine (Mycillin Sol Meiji, Meiji Seika Pharma, Osaka, Japan, 0.05 ml/kg). After the surgery, the testes and epididymides from both sides were separated and weighed.

Hormone analysis

INSL3 assay: Plasma INSL3 concentrations were measured by a time-resolved fluorescence immunoassay (TRFIA) without an extraction procedure [20]. Briefly, microtitration plates for the TRFIA (PerkinElmer, Wallac Oy, Finland) were coated with 100 µl per well of anti-mouse IgG goat polyclonal antibody (KPL; 5 µg/ml in 0.05 M sodium bicarbonate; pH 9.7) for 2 h at room temperature. The wells were then washed three times with 300 µl of 0.15 M sodium chloride and 200 µl of DELFIA assay buffer (PerkinElmer) was added and the plates placed at 4°C overnight. Prior to the assay, each bull plasma sample was diluted four times with DELFIA assay buffer. The wells were drained, and 50 µl of synthetic bovine INSL3 [21] for the standards, or 50 µl of the samples, plus 50 µl of anti-bovine INSL3 mouse monoclonal antibody [21] (2-8F, dilution 1:1,000,000 in DELFIA assay buffer) was added to each well. Biotin-labeled canine INSL3 [12] (2 ng/ml in DELFIA Assay Buffer) was added and the plates incubated for 1 h at room temperature. The wells were then washed three times with 300 µl DELFIA wash buffer (PerkinElmer), 100 µl of Eu-labeled streptavidin (100 ng/ml in DELFIA assay buffer; PerkinElmer) was added and the plates were incubated for 30 min at room temperature. After washing five times with 300 µl of the wash buffer, 100 µl of enhancement solution (PerkinElmer) was dispensed in each well, followed by shaking at 80 rpm for 15 min at room temperature. Finally, time-resolved fluorescence was measured with an ARVO multilabel counter (PerkinElmer). The minimum detection limit of the INSL3 TRFIA was 0.156 ng/ml, and detection was reliable in the range of 0.156 to 20 ng/ml. The intra-assay and inter-assay coefficients of variation (CVs) for plasma samples of the cattle were 3.6% (n = 4) and 3.9% (n = 7), respectively.

Testosterone assay: Plasma testosterone concentrations were measured by the enzyme immunoassay (EIA) established in our laboratory [11, 22]. Eight-well strips (Corning Inc. Life Sciences, Lowell, MA, USA) were coated with 100 µl per well of anti-rabbit IgG goat polyclonal antibody (KPL, Gaithersburg, MD, USA; 5 µg/ml in 0.05 M sodium bicarbonate; pH 9.7) for 2 h at room temperature. The wells were then washed three times with 300 µl of 0.15 M sodium chloride. Next, 200 µl of assay buffer (0.01 M phosphate buffer containing 0.15 sodium chloride, pH 7.4) supplemented with 0.1% bovine serum albumin (BSA: Cohn Fraction V, Sigma-Aldrich, St. Louis, MO, USA) and 0.02% ProClin 950 (Sigma-Aldrich) was added and the plates kept overnight at 4°C to block areas of the well that were not coated with antibody. Various concentrations of testosterone standards were diluted with the assay buffer. Next, 125 µl of standards or bovine plasma samples was extracted with 2 ml of diethyl ether and the dried extract was dissolved in 125 µl of the assay buffer by vigorous vortexing. The wells were drained, followed by the addition of 50 µl of extracted testosterone standards or extracted samples, plus 50 µl of the HRP-labeled testosterone (Cosmo Bio, Tokyo, Japan; 1:1,500 dilution in the assay buffer) and 50 µl of anti-testosterone rabbit polyclonal antibody (Cosmo Bio; 1:400,000 dilution in the assay buffer). The mixture was then incubated for 2 h at room temperature. After the reaction, the wells were washed three times with 400 µl of wash buffer and 100 µl of substrate containing 3, 3', 5, 5'-tetramethylbenzidine (Sigma-Aldrich) solution was added and incubated for 30 min at room temperature. The reaction was stopped by adding 100 µl of 2 M sulfuric acid, and the optical density was measured at 450 nm using an xMark microplate absorbance spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). The minimum detection limit was 0.156 ng/ml, and the reliable detection limit was 0.156 to 20 ng/ml. The intra-assay and

inter-assay CVs were 6.1% (n = 4) and 3.0% (n = 6), respectively.

Inhibin assay: Plasma inhibin concentrations were measured by the TRFIA [20]. Bovine inhibin standards (Tanpaku Seisei Kougyou, Isesaki, Japan), anti-bovine inhibin antibody (Tanpaku Seisei Kougyou), and biotinylated bovine inhibin were used for the assay. The minimum detection limit of the inhibin TRFIA was 0.312 ng/ml and the assay detection range was from 0.312 to 20 ng/ml. The intra-assay and inter-assay CVs were 1.9% (n = 4) and 18.5% (n = 7), respectively.

IGF-I assay: Extraction of IGF-I from bovine plasma was performed using trifluoroacetic acid and acetonitrile as previously described [20]. Plasma IGF-I concentrations were measured by the EIA [20]. Human IGF-I standards (NIDDK no. 01), anti-human IGF-I rabbit serum (NIDDK no. AFP4892898), and horseradish peroxidase-labeled IGF-I were used for the assay. The minimum detection limit of the IGF-I EIA was 3.125 ng/ml, and the detection was reliable in the range of 3.125–400 ng/ml. The intra-assay and inter-assay CVs were 3.9% (n = 4) and 9.6% (n = 6), respectively.

Data analysis

Body weight was not measured in two calves at 1 month, one calf at 2 months, six calves at 5 months and one calf at 6 months; therefore, the total number data points for body weight data was 150. Scrotal circumference was not measured in two calves at 1 month, one calf at 2 months, and one calf at 5 months, and the total number of data points for scrotal circumference data was 136. Blood samples were not taken from one calf at 0 month, and the total number of blood samples was 159. We investigated the effect of age (month) on body weight, scrotal circumference, and hormonal concentrations by conducting a two-way ANOVA analysis using the Generalized Linear Models (GLMs) procedure of the SPSS version 24 software (IBM, Somers, NY, USA). *Post-hoc* pairwise comparisons were made by the Bonferroni correction for differences between two time-points. The correlations between scrotal circumference and hormonal concentrations were analyzed using the curve estimation procedure of Regression Analysis of the SPSS version 24 software. The effect of testicular weight at 7 months of age (heavier: > 60 g *vs*. lighter: < 60 g) on hormonal concentrations and scrotal circumference at each month (0 to 7 months) was also examined by two-way ANOVA with the GLMs procedure to investigate any associations between hormonal secretion and testicular development. The threshold value of testicular weight (60 g) was determined based on the distribution of the individual data (there was a clear gap at 60 g between heavier (n = 14) and lighter (n = 6) groups). The *post-hoc* pairwise comparisons were performed by the least significant difference test for differences between the heavier and lighter testes at a specific time point. The data are expressed as mean ± standard error of the mean (SEM). Differences were considered significant at $P < 0.05$.

Results

There were significant effects of age on the body weight and scrotal circumference of calves ($P < 0.0001$). Body weight and scrotal circumference increased gradually until the end of sampling at 7 months (Fig. 1A), showing higher ($P < 0.05$) values than those from previous months.

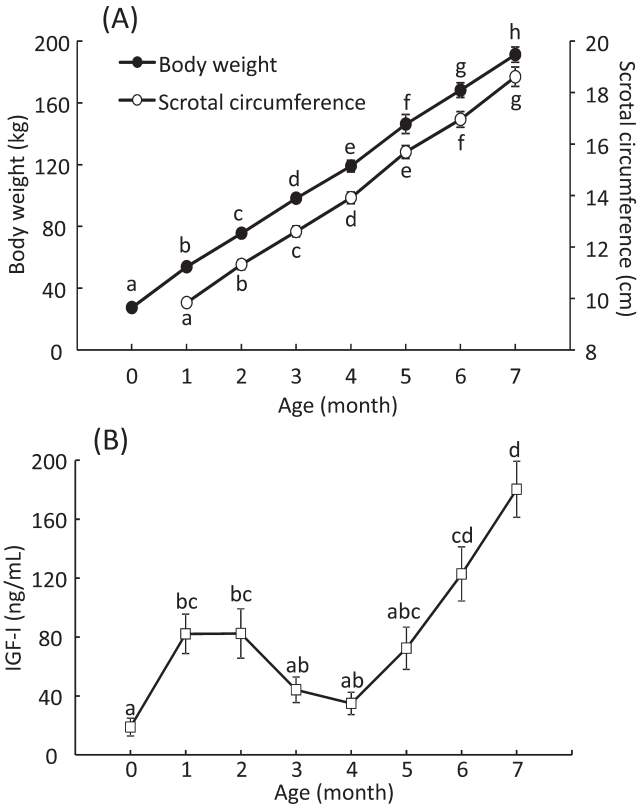


Fig. 1. Changes in body weight and scrotal circumference (A) and plasma IGF-I concentrations (B) in bull calves from 0 to 7 months of age. Data are expressed as a mean ± SEM. Numbers of body weight data were 20 at 0, 3, 4, and 7 months; 18 at 1 month; 19 at 2 months; 14 at 5 months; and 19 at 6 months. Numbers of scrotal circumference data were 20 at 0, 3, 4, 6, and 7 months; 18 at 1 month; and 19 at 2 and 5 months. Numbers of IGF-I data were 19 at 0 month and 20 from 1 to 7 months. Differing superscripts between two time points within each measurement (body weight, scrotal circumference or IGF-I concentration) indicate differences ($P < 0.05$).

There was a significant effect of age on plasma IGF-I concentrations in the bull calves ($P < 0.0001$). Plasma IGF-1 concentrations rose from 0 to 1 month (Fig. 1B, $P < 0.05$) and then did not change from 1 to 2 months; concentrations at 3 and 4 months did not differ significantly from month 0, indicative of a small transient increase of IGF-I at 1 and 2 months of age. IGF-I concentrations then increased progressively from 4 to 7 months (4 *vs*. 6 months, 5 *vs*. 7 months, $P < 0.05$).

There were significant effects of age on plasma INSL3 and testosterone concentrations in the bull calves ($P < 0.0001$). The plasma INSL3 concentrations increased progressively from 0 to 7 months (Fig. 2 A, 0 *vs*. 1 month, 1 *vs*. 3 months, 3 *vs*. 7 months, $P < 0.05$). Plasma testosterone concentrations did not change significantly from 0 to 4 months (Fig. 2A), indicating no clear rise in the levels of the hormone until 4 months. Testosterone concentrations increased from 0 to 5 months and from 5 and 7 months ($P < 0.05$).

There was a significant effect of age on plasma inhibin concentra-

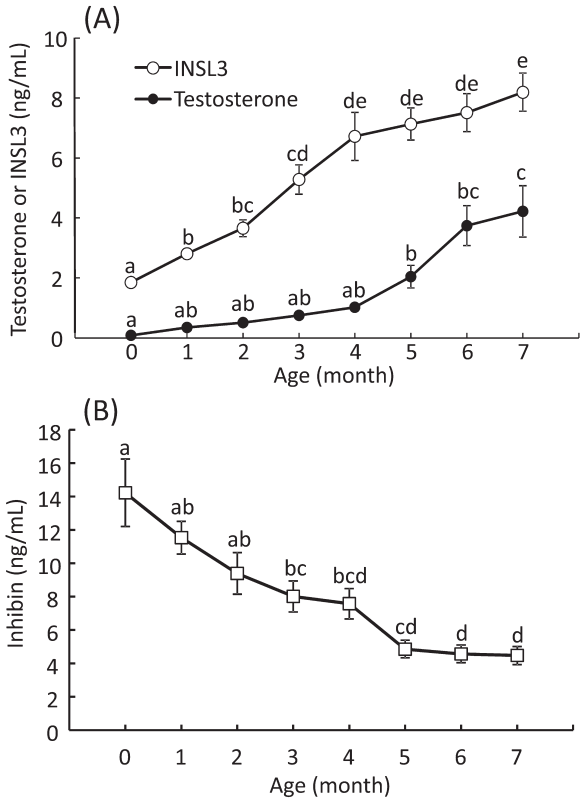


Fig. 2. Changes in plasma INSL3 and testosterone concentrations (A) and plasma inhibin concentrations (B) in bull calves from 0 to 7 months of age. Data are expressed as a mean ± SEM. Numbers of hormonal data were 19 at 0 month and 20 from 1 to 7 months. Differing superscripts between two time points for each hormone indicate differences ($P < 0.05$).

tions in the bull calves ($P < 0.0001$). The plasma inhibin concentrations decreased from 0 to 3 months and from 3 to 6 and 7 months (Fig. 2B, $P < 0.05$), implying a constant decline in the levels of the hormone during the whole period.

The plasma INSL3, testosterone, and IGF-I concentrations in the bull calves were positively correlated with the scrotal circumference from 1 to 7 months (Fig. 3A–C), whereas a negative correlation was observed between inhibin concentrations and scrotal circumference during the same period (Fig. 3D). The correlation coefficients were highest for INSL3 ($r = 0.647$, $n = 136$, $P < 0.0001$), followed by testosterone ($r = 0.597$, $n = 136$, $P < 0.0001$), inhibin ($r = –0.453$, $n = 136$, $P < 0.0001$), and IGF-I ($r = 0.400$, $n = 136$, $P < 0.0001$).

The plasma INSL3 concentrations in the bull calves tended to be positively correlated with the testicular weight at 7 months ($r = 0.407$, $n = 20$, $P = 0.08$), but the other three hormonal concentrations showed no correlation with the testicular weight (IGF-I, $r = 0.215$, $P > 0.15$; testosterone, $r = 0.175$, $P > 0.15$; inhibin, $r = 0.044$, $n = 20$, $P > 0.15$). When calves were classified based on the testicular weight at 7 months into heavier (> 60 g) or lighter (< 60 g), the plasma IGF-I concentrations were lower at 6 months in the calves with smaller testes than the calves with heavier testes (Fig. 4A, $P < 0.05$). The bull calves with lighter testes have lower plasma INSL3 concentrations from 3 to 7 months and testosterone at 5 and 7 months compared to the bull calves with heavier testes (Fig. 4B and C, $P < 0.05$). The plasma inhibin concentrations were lower from 1 to 4 months in the calves with lighter testes than the calves with heavier testes (Fig. 4D, $P < 0.05$). The scrotal circumference was smaller from 3 to 7 months in the calves with lighter testes than the calves with heavier testes (Fig. 4E, $P < 0.05$).

Discussion

Associations between testicular size and peripheral levels of testicular and metabolic hormones during development have not been elucidated in Japanese Black beef bull calves. In the present study, plasma INSL3, testosterone, and IGF-I concentrations were positively correlated with the scrotal circumference. The roles of testosterone in testicular development and function have been well characterized in bulls [30, 31]. In addition, the testicular Leydig cell-derived hormone, INSL3, has been reported to stimulate spermatogenesis in rats [4] and pigs [5]. Higher levels of nutrition during calfhood were shown to increase serum IGF-I concentrations and testicular size at pre-puberty in beef bulls [19], indicating a close relationship between peripheral blood levels of IGF-I and testicular development. Thus, the increments of these three hormonal concentrations during calfhood in the present study may have stimulated testicular development in the beef bull calves.

Conversely, a negative correlation between plasma inhibin concentrations and the scrotal circumference was observed during the developmental period in the bull calves in the present study, mainly due to the decline in inhibin levels during this period. It has been reported that blood inhibin concentrations decline during the developmental period in bull calves [17, 32] even though total amounts of testicular inhibin increase [33]. It has been speculated that the decrease in circulating inhibin concentration at this stage is associated with the formation of the blood-testis barrier and the blockage of transfer of the protein from seminiferous tubules to blood vessels [33].

In the current study, the correlation between testicular size (scrotal circumference and testicular weight) and plasma concentration of INSL3 was the highest among the 4 hormones studied. This suggests that the peripheral blood concentration of INSL3 may be the preferred functional indicator for total testicular volume in bull calves before puberty. In this context, it was previously demonstrated that scrotal circumference showed a higher correlation with plasma INSL3 than with testosterone concentrations during puberty and post-puberty [13]. INSL3 is secreted in pulses into circulating blood in bulls and bucks in response to luteinizing hormone (LH), but the amplitude of INSL3 pulses is much lower than that of testosterone [13, 27–29]; this suggests that INSL3 may be a more stable marker than testosterone for single time point blood sample measurements for the evaluation of total Leydig cell count per pair of testes. It is unclear why the correlation coefficient value was lower for inhibin concentrations with the scrotal circumferences than INSL3 and testosterone; however, it could be related to the formation of the blood-testicular barrier which restricts the immediate release of inhibin from Sertoli cells to circulating blood during development [33].

We compared the hormonal concentration and scrotal circumference

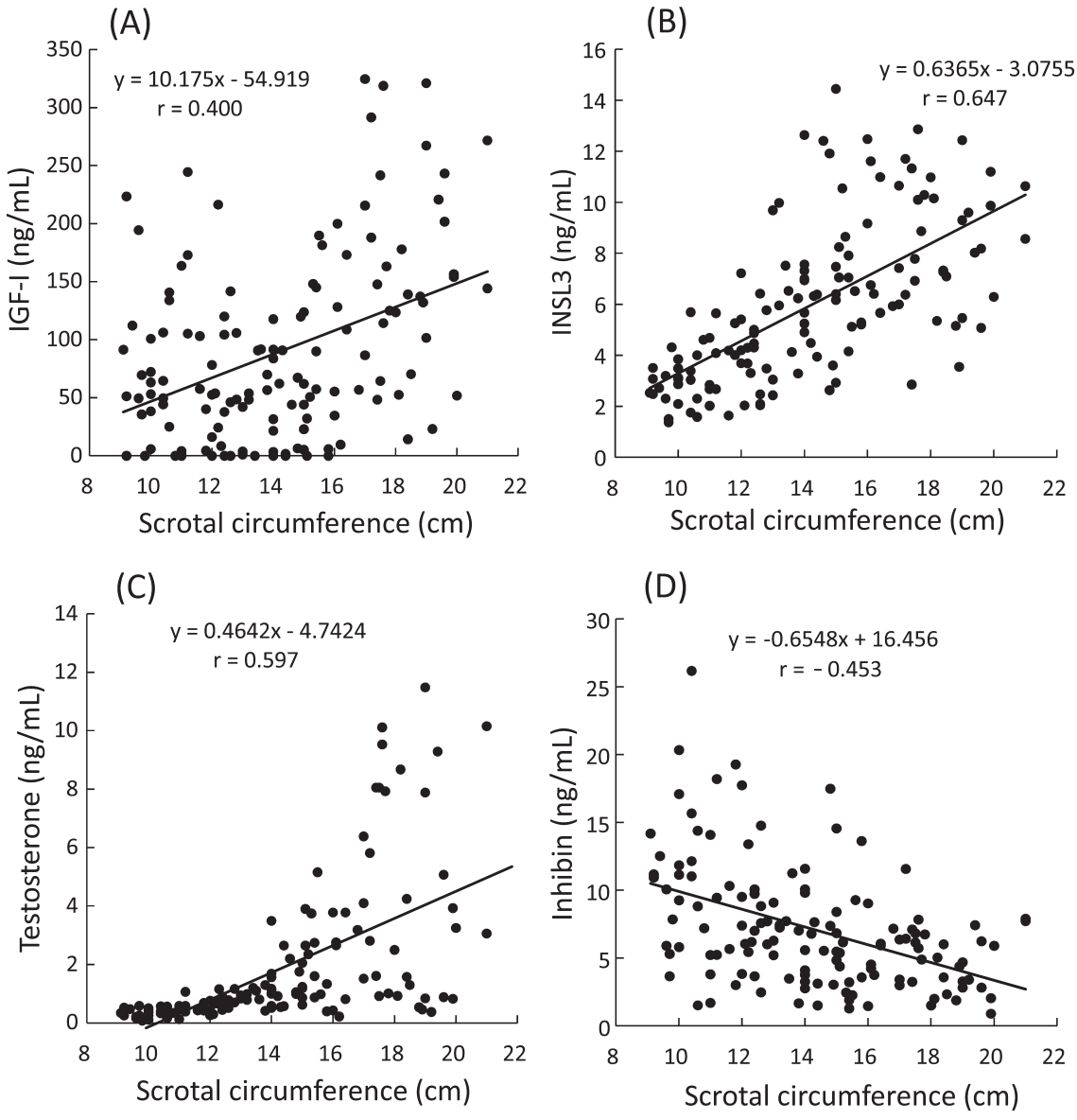
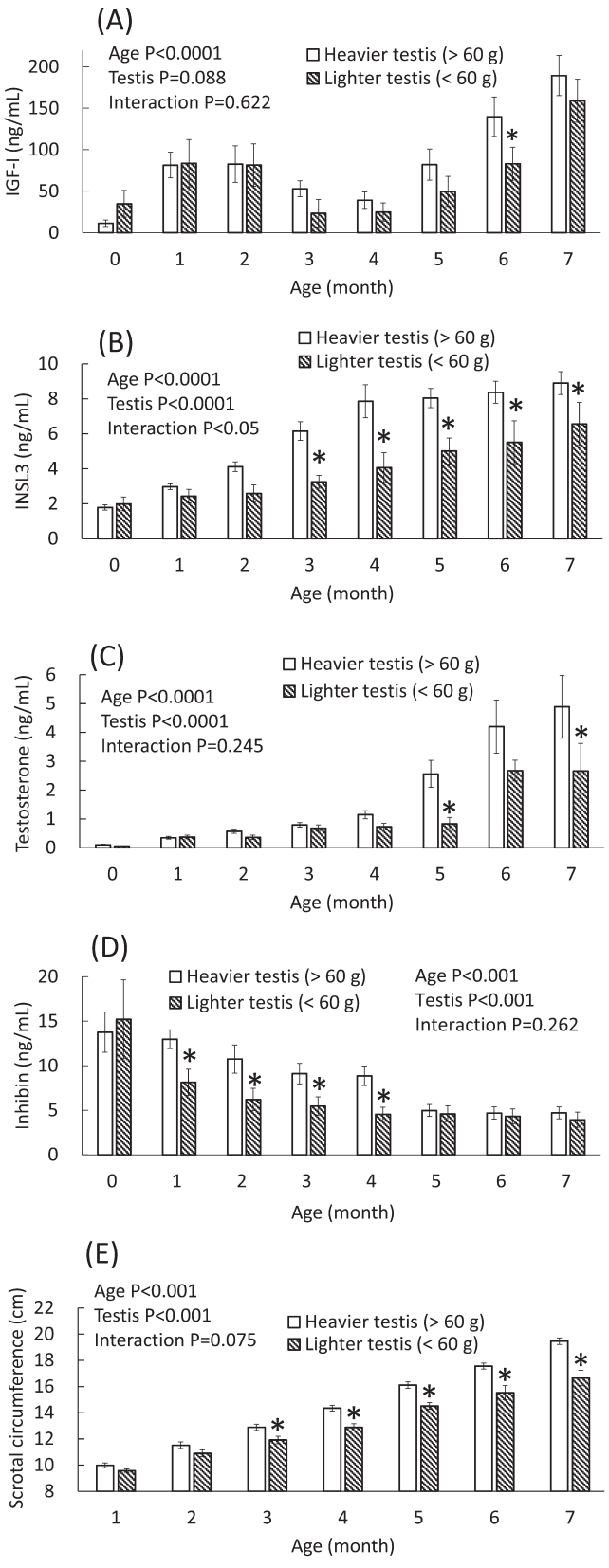


Fig. 3. Correlations between plasma IGF-I (A), INSL3 (B), testosterone (C), and inhibin (D) concentrations and scrotal circumference in bull calves from 1 to 7 months of age (n = 136). An equation of linear regression and an r value are shown for each figure.

measurements taken each month, from birth to 7 months of age, between calves with heavier (> 60 g) and lighter (< 60 g) testes at 7 months to examine whether testicular size at pre-puberty (7 months) can be predicted by hormonal concentrations or scrotal circumference in early calfhood (0 to 6 months). In this study, we found differences in the concentrations of inhibin (1 to 4 months), INSL3 (3 to 6 months), testosterone (5 months), and IGF-I (6 months), as well as in scrotal circumference (3 to 6 months), between the calves with heavier and those with lighter testes. These results suggest that plasma inhibin and INSL3 concentrations in early calfhood may be functional predictors of total testicular weight at pre-puberty. Calves with larger testicular size at pre-puberty showed larger testes and higher sperm production post-puberty when fed a high level of nutrition during calfhood [18, 19].

In the present study, plasma IGF-I concentrations increased transiently during the first 2 months after birth in the beef bull calves, followed by a relative decrease at 3 and 4 months and then a steep rise from 4 to 7 months of age. Such a pattern of changes in blood IGF-I concentrations during the growing phase in bull calves had not been observed in previous reports [23–25]. It has been suggested that plasma IGF-I concentrations of neonatal calves are affected by the amount, time point, and feeding frequency of colostrum [26]. We propose that calf starter pellets containing elevated amounts of crude protein given to bull calves from 1 week until 2.5 months of



age may induce the transient small rise of plasma IGF-I concentrations, and that replacement with ordinary concentrate may cause the reduction at 3 and 4 months. In a previous report [20], plasma IGF-I concentrations did not increase noticeably from 4 to 7 months in the Japanese Black beef bull calves. It is unclear why the change in IGF-I concentrations differed between our previous and present studies. In this study, we collected blood samples monthly from identical calves from 4 to 7 months of age, whereas we used different calves during the same period in the previous study, which may explain the differing results observed.

Plasma INSL3 concentrations increased continuously from birth to pre-puberty, while testosterone concentrations increased only in the latter phase in Japanese Black beef bull calves, suggesting differential regulation of the two hormonal secretions from testicular Leydig cells in ruminants [27–29]. In this study, plasma testosterone concentrations did not increase significantly from 0 to 3 months but rose significantly during the same period in the Japanese Black beef bull calves in the previous study [11]. Although the reasons for the discrepancy are unclear, differences in weaning age may affect testosterone secretion (2 days previously versus 5 months in the present study).

The results of this study confirmed the decrease in blood inhibin concentrations during the developmental period after birth in Holstein [17] and Japanese Black beef [32] bull calves. However, it was shown that the inhibin concentration increased for the initial 3 months after birth and then decreased until 10 months in plasma samples collected weekly from Holstein bull calves [15, 33]. In addition, the plasma inhibin concentrations were higher at several time points in this study than those previously reported [15, 17, 32, 33]. It is unclear why such inconsistencies occurred in the changes and values of inhibin concentrations in the 3 months after birth in bull calves. However, differences in sampling intervals, type of breed, and method of immunoassay (e.g., biotinylated inhibin was used as a labeled hormone in this study) may help to explain the discrepancies.

In conclusion, secretions of INSL3, testosterone, inhibin, and IGF-I are associated with testicular development in Japanese Black beef bull calves. Plasma INSL3 concentrations may be the best functional indicator among these hormones for total testicular volume in bull calves. Furthermore, INSL3 and inhibin concentrations in early calfhood may be functional predictors for total testicular weight in pre-puberty.

Fig. 4. Changes of plasma IGF-I (A), INSL3 (B), testosterone (C), and inhibin (D) concentrations from 0 to 7 months of age and scrotal circumference (E) from 1 to 7 months in bull calves with lighter (< 60 g) and heavier (> 60 g) testicular weight at 7 months. Data are expressed as a mean ± SEM. Number of hormonal data was 6 from 0 to 7 months for calves with small testicular weight. Numbers of hormonal data were 13 at 0 month and 14 from 1 to 7 months for calves with heavier testicular weight. Number of scrotal circumference data was 6 from 1 to 7 months for calves with lighter testicular weight. Numbers of scrotal circumference data were 12 at 1 month, 13 at 2 months and 14 from 3 to 7 months for calves with heavier testicular weight. * $P < 0.05$ compared to calves with heavier (> 60 g) testes.

Acknowledgements

We wish to thank Dr Erika E. Büllesbach, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, USA for providing the anti-bovine INSL3 mouse monoclonal antibody (2-8F) and bovine INSL3. The authors are grateful to NIDDK, NIH, and Dr AF Parlow, National Hormone and Pituitary Program, Harbor-UCLA Medical Center, for providing the hIGF-I (Lot no. 1). This study was partly supported by Grants-in-Aid for Scientific Research (C) of the Japanese Society for the Promotion of Science (Grant number 16K08058).

References

1. **Ivell R, Bathgate RAD.** Reproductive biology of the relaxin-like factor (RLF/INSL3). *Biol Reprod* 2002; **67**: 699–705. [Medline] [CrossRef]
2. **Nef S, Parada LF.** Cryptorchidism in mice mutant for Insl3. *Nat Genet* 1999; **22**: 295–299. [Medline] [CrossRef]
3. **Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayernia K, Holstein AF, Engel W, Adham IM.** Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. *Mol Endocrinol* 1999; **13**: 681–691. [Medline] [CrossRef]
4. **Kawamura K, Kumagai J, Sudo S, Chun SY, Pisarska M, Morita H, Toppari J, Fu P, Wade JD, Bathgate RA, Hsueh AJ.** Paracrine regulation of mammalian oocyte maturation and male germ cell survival. *Proc Natl Acad Sci USA* 2004; **101**: 7323–7328. [Medline] [CrossRef]
5. **Sagata D, Minagawa I, Kohriki H, Pitia AM, Uera N, Katakura Y, Sukigara H, Terada K, Shibata M, Park EY, Hasegawa Y, Sasada H, Kohsaka T.** The insulin-like factor 3 (INSL3)-receptor (RXFP2) network functions as a germ cell survival/anti-apoptotic factor in boar testes. *Endocrinology* 2015; **156**: 1523–1539. [Medline] [CrossRef]
6. **Büllesbach EE, Rhodes R, Rembiesa B, Schwabe C.** The relaxin-like factor is a hormone. *Endocrine* 1999; **10**: 167–169. [Medline] [CrossRef]
7. **Ferlin A, Garolla A, Rigon F, Rasi Caldogno L, Lenzi A, Foresta C.** Changes in serum insulin-like factor 3 during normal male puberty. *J Clin Endocrinol Metab* 2006; **91**: 3426–3431. [Medline] [CrossRef]
8. **Johansen ML, Anand-Ivell R, Mouritsen A, Hagen CP, Mieritz MG, Søeborg T, Johannsen TH, Main KM, Andersson AM, Ivell R, Juul A.** Serum levels of insulin-like factor 3, anti-Mullerian hormone, inhibin B, and testosterone during pubertal transition in healthy boys: a longitudinal pilot study. *Reproduction* 2014; **147**: 529–535. [Medline] [CrossRef]
9. **Boockfor FR, Fullbright G, Büllesbach EE, Schwabe C.** Relaxin-like factor (RLF) serum concentrations and gubernaculum RLF receptor display in relation to pre- and neonatal development of rats. *Reproduction* 2001; **122**: 899–906. [Medline] [CrossRef]
10. **Anand-Ivell R, Heng K, Hafen B, Setchell B, Ivell R.** Dynamics of INSL3 peptide expression in the rodent testis. *Biol Reprod* 2009; **81**: 480–487. [Medline] [CrossRef]
11. **Kawate N, Ohnari A, Pathirana IN, Sakase M, Büllesbach EE, Takahashi M, Inaba T, Tamada H.** Changes in plasma concentrations of insulin-like peptide 3 and testosterone from birth to pubertal age in beef bulls. *Theriogenology* 2011; **76**: 1632–1638. [Medline] [CrossRef]
12. **Pathirana IN, Yamasaki H, Kawate N, Tsuji M, Büllesbach EE, Takahashi M, Hatoya S, Inaba T, Tamada H.** Plasma insulin-like peptide 3 and testosterone concentrations in male dogs: changes with age and effects of cryptorchidism. *Theriogenology* 2012; **77**: 550–557. [Medline] [CrossRef]
13. **Hannan MA, Kawate N, Fukami Y, Weerakoon WWPN, Büllesbach EE, Inaba T, Tamada H.** Changes of plasma concentrations of insulin-like peptide 3 and testosterone, and their association with scrotal circumference during pubertal development in male goats. *Theriogenology* 2017; **92**: 51–56. [Medline] [CrossRef]
14. **Kastelic JP.** Understanding and evaluating bovine testes. *Theriogenology* 2014; **81**: 18–23. [Medline] [CrossRef]
15. **Kaneko H, Noguchi J, Kikuchi K, Akagi S, Shimada A, Taya K, Watanabe G, Hasegawa Y.** Production and endocrine role of inhibin during the early development of bull calves. *Biol Reprod* 2001; **65**: 209–215. [Medline] [CrossRef]
16. **Kaneko H, Noguchi J, Kikuchi K, Hasegawa Y.** Molecular weight forms of inhibin a and inhibin B in the bovine testis change with age. *Biol Reprod* 2003; **68**: 1918–1925. [Medline] [CrossRef]
17. **Matsuzaki S, Uenoyama Y, Okuda K, Watanabe G, Kitamura N, Taya K, Cruzana MB, Yamada J.** Prepubertal changes in immunoreactive inhibin concentration in blood serum and testicular tissue in Holstein bull calves. *J Vet Med Sci* 2001; **63**: 1303–1307. [Medline] [CrossRef]
18. **Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews DH Jr, Mir PS, Kastelic JP.** Effect of nutrition during calfhood and peripubertal period on serum metabolic hormones, gonadotropins and testosterone concentrations, and on sexual development in bulls. *Domest Anim Endocrinol* 2007; **33**: 1–18. [Medline] [CrossRef]
19. **Brito LF, Barth AD, Rawlings NC, Wilde RE, Crews DH Jr, Mir PS, Kastelic JP.** Effect of improved nutrition during calfhood on serum metabolic hormones, gonadotropins, and testosterone concentrations, and on testicular development in bulls. *Domest Anim Endocrinol* 2007; **33**: 460–469. [Medline] [CrossRef]
20. **Weerakoon WWPN, Sakase M, Kawate N, Hannan MA, Kohama N, Tamada H.** Plasma IGF-I, INSL3, testosterone, inhibin concentrations and scrotal circumferences surrounding puberty in Japanese Black beef bulls with normal and abnormal semen. *Theriogenology* 2018; **114**: 54–62. [Medline] [CrossRef]
21. **Büllesbach EE, Schwabe C.** The primary structure and the disulfide links of the bovine relaxin-like factor (RLF). *Biochemistry* 2002; **41**: 274–281. [Medline] [CrossRef]
22. **Kibushi M, Kawate N, Kaminogo Y, Hannan MA, Weerakoon WW, Sakase M, Fukushima M, Seyama T, Inaba T, Tamada H.** Fetal gender prediction based on maternal plasma testosterone and insulin-like peptide 3 concentrations at midgestation and late gestation in cattle. *Theriogenology* 2016; **86**: 1764–1773. [Medline] [CrossRef]
23. **Stick DA, Davis ME, Loerch SC, Simmen RC.** Relationship between blood serum insulin-like growth factor I concentration and postweaning feed efficiency of crossbred cattle at three levels of dietary intake. *J Anim Sci* 1998; **76**: 498–505. [Medline] [CrossRef]
24. **Graham TW, Breher JE, Farver TB, Cullor JS, Kehrli ME Jr, Oberbauer AM.** Biological markers of neonatal calf performance: the relationship of insulin-like growth factor-I, zinc, and copper to poor neonatal growth. *J Anim Sci* 2010; **88**: 2585–2593. [Medline] [CrossRef]
25. **Gutiérrez V, Espasandin AC, Astessiano AL, Casal A, López-Mazz C, Carriquiry M.** Calf foetal and early life nutrition on grazing conditions: metabolic and endocrine profiles and body composition during the growing phase. *J Anim Physiol Anim Nutr (Berl)* 2013; **97**: 720–731. [Medline] [CrossRef]
26. **Hammon HM, Steinhoff-Wagner J, Schönhusen U, Metges CC, Blum JW.** Energy metabolism in the newborn farm animal with emphasis on the calf: endocrine changes and responses to milk-born and systemic hormones. *Domest Anim Endocrinol* 2012; **43**: 171–185. [Medline] [CrossRef]
27. **Hannan MA, Fukami Y, Kawate N, Sakase M, Fukushima M, Pathirana IN, Büllesbach EE, Inaba T, Tamada H.** Plasma insulin-like peptide 3 concentrations are acutely regulated by luteinizing hormone in pubertal Japanese Black beef bulls. *Theriogenology* 2015; **84**: 1530–1535. [Medline] [CrossRef]
28. **Hannan MA, Kawate N, Fukami Y, Pathirana IN, Büllesbach EE, Inaba T, Tamada H.** Acute regulation of plasma insulin-like peptide 3 concentrations by luteinizing hormone in male goats. *Theriogenology* 2016; **86**: 749–756. [Medline] [CrossRef]
29. **Hannan MA, Kawate N, Fukami Y, Weerakoon WW, Büllesbach EE, Inaba T, Tamada H.** Effects of long-acting GnRH antagonist, degarelix acetate, on plasma insulin-like peptide 3, testosterone and luteinizing hormone concentrations, and scrotal circumference in male goats. *Theriogenology* 2017; **88**: 228–235. [Medline] [CrossRef]
30. **Amann RP.** Endocrine changes associated with onset of spermatogenesis in Holstein bulls. *J Dairy Sci* 1983; **66**: 2606–2622. [Medline] [CrossRef]
31. **Rawlings N, Evans ACO, Chandolia RK, Bagu ET.** Sexual maturation in the bull. *Reprod Domest Anim* 2008; **43**(Suppl 2): 295–301. [Medline] [CrossRef]
32. **Miyamoto A, Umezu M, Ishii S, Furusawa T, Masaki J, Hasegawa Y, Hota M.** Serum inhibin, FSH, LH and testosterone levels and testicular inhibin content in beef bulls from birth to puberty. *Anim Reprod Sci* 1989; **20**: 165–178. [CrossRef]
33. **Kaneko H, Matsuzaki M, Noguchi J, Kikuchi K, Ohnuma K, Ozawa M.** Changes in circulating and testicular levels of inhibin A and B during postnatal development in bulls. *J Reprod Dev* 2006; **52**: 741–749. [Medline] [CrossRef]