

# Plasma insulin-like peptide 3 concentrations are acutely regulated by luteinizing hormone in pubertal Japanese Black beef bulls

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1 Acute regulation of insulin-like peptide 3 secretion in peripheral blood  
2 by LH in pubertal Japanese Black beef bulls

3

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21 **Abstract**

22 Insulin-like peptide 3 (INSL3) is a major secretory product of testicular Leydig cells.  
23 The mechanism of acute regulation of INSL3 secretion is still unknown. The present  
24 study was undertaken in pubertal beef bulls to: (1) determine the temporal relationship  
25 of pulsatile secretion among LH, INSL3 and testosterone; and (2) monitor acute  
26 regulation of INSL3 secretion by LH using GnRH analogue and hCG. Blood samples  
27 were collected from Japanese Black beef bulls (n=6) at 15-min intervals for 8 h.  
28 Moreover, blood samples were collected after GnRH (-0.5 h, 0 h, 1 h, 2 h, 3 h, 4 h, 5 h,  
29 and 6 h) and after hCG (-0.5 h, 0 h, 2 h, 4 h, 8 h, day 1, day 2, day 4, day 8 and day 12)  
30 treatments. Concentrations of LH, INSL3 and testosterone determined by enzyme-  
31 immunoassays (EIA) indicated that secretion in the general circulation was pulsatile.  
32 The frequency of LH, INSL3 and testosterone pulses was  $4.7 \pm 0.9$ ,  $3.8 \pm 0.2$  and  $1.0 \pm$   
33  $0.0$ , respectively, during the 8 h period. Seventy percent of these INSL3 pulses peaked  
34 within 1 h after a peak of an LH pulse had occurred. The mean increasing rate  
35 (peak/basal concentration) of testosterone pulses was higher ( $P < 0.001$ ) than those of  
36 INSL3 pulses. After GnRH treatment, LH concentrations increased ( $P < 0.01$ )  
37 dramatically 1 h post-treatment and remained high ( $P < 0.01$ ) until 5 h, while an elevated  
38 ( $P < 0.05$ ) INSL3 concentration was observed at 1 h, 2 h and 6 h after treatment.

39 Testosterone concentrations increased ( $P<0.01$ ) 1 h after the treatment and remained  
40 high till the end of sampling. After hCG treatment, an increase of INSL3 concentration  
41 was observed at 2 h, 4 h, day 2, day 4 and day 8 of treatment ( $P<0.05$ ), whereas in case  
42 of testosterone, concentrations remained significantly ( $P<0.01$ ) high till 8 day after  
43 treatment. The increasing rate (maximum/pre-treatment concentration) of testosterone  
44 concentrations after injecting GnRH or hCG was much higher ( $P<0.001$ ) than that of  
45 INSL3. Our results demonstrate that the secretory pattern of INSL3 in the peripheral  
46 blood is pulsatile in bull and that endogenous and exogenous LH can stimulate INSL3  
47 secretion soon after the treatment. This suggests an acute regulation of INSL3 by LH in  
48 beef bulls. Moreover, the increasing rate of INSL3 pulses are much smaller than those  
49 of testosterone pulses, and therefore INSL3 can be used as a less-fluctuating marker  
50 than testosterone to evaluate functions of testicular Leydig cells in the pubertal beef  
51 bulls.

52 **Keywords:** INSL3; LH; Testosterone; GnRH; HCG; Beef bull

53 **1. Introduction**

54           Insulin-like peptide 3 (INSL3) is a major secretory product of testicular Leydig  
55 cells in all mammalian species examined so far [1–2]. The two main known functions of  
56 INSL3 in the male are the endocrine regulatory effect involved in completing the  
57 trans-abdominal phase of testicular descent in mice [3, 4] and a paracrine function  
58 exhibiting an anti-apoptotic effect to protect male germ cells in rats [5]. According to  
59 studies on human, secretion of INSL3 is related to the differentiation status of testicular  
60 Leydig cells and is stimulated by the long-term trophic effects of LH [1, 6–9]. However,  
61 the process of acute regulation of INSL3 secretion is mostly unknown. Detection of  
62 INSL3 in the peripheral blood of humans [7, 10, 11], dogs [12], and cattle [13] indicate  
63 that INSL3 may have additional endocrine effects in mammalian male species.  
64 According to recent studies in our laboratory, dynamics of secretory patterns of INSL3  
65 and testosterone in peripheral plasma are different during sexual development in male  
66 dogs [12] and beef bulls [13], although both hormones are secreted from the unique  
67 source of testicular Leydig cells. It is well documented that in many species including  
68 bull [14, 15] secretion of LH occurred in a pulsatile manner stimulating testicular  
69 Leydig cells to produce pulsatile secretion of testosterone. However, under  
70 physiological conditions the pulsatile secretory pattern of INSL3 and its relation with

71 LH has not been elucidated.

72           Endogenous LH increased by gonadotropin releasing hormone (GnRH) or human  
73 chorionic gonadotropin (hCG), which possesses LH activity, caused a significant  
74 increase of testosterone in the general circulation of bulls [16–19], male goats [20], and  
75 rams [21]. In men testosterone concentrations in peripheral blood taken daily for 8 days  
76 increased after hCG treatment while INSL3 concentrations did not change [22]. It  
77 remains unknown whether endogenous and exogenous LH can acutely regulate the  
78 secretion of INSL3 in domestic animals.

79           The objectives of this paper are: (1) to determine the temporal relationship of  
80 pulsatile secretion among LH, INSL3 and testosterone; and (2) to monitor acute  
81 regulation of INSL3 secretion by LH using GnRH analogue and hCG in pubertal beef  
82 bulls.

83

## 84 **2. Materials and methods**

### 85 *2.1. Animals*

86           Japanese Black beef bulls (n=6, aged 10–19 mo) raised in an experimental beef  
87 cattle station in the Northern Center of Agriculture Technology of Hyogo Prefecture in  
88 Japan were used for the present study. The selected beef bulls had no apparent

89 abnormalities of the reproductive status and testicular presence was checked manually  
90 to confirm the presence of both testes inside the scrotum. These bulls remained normal  
91 in appearance and health during all experiments. Bulls were kept under natural light in  
92 an open shelter covered by a roof and were maintained by ad libitum hay and  
93 concentrate to meet or exceed Japanese Feeding Standard recommendations for the beef  
94 bulls.

95

## 96 *2.2. Experiment 1*

97 Experiment 1 was done to determine the temporal relationship among INSL3, LH  
98 and testosterone at 15-min intervals sampling for an 8 h session in beef bulls (aged,  
99 10–11 mo; n=6). Blood sampling for all bulls was started at 10:00 AM and ended at  
100 6:00 PM. An indwelling jugular venous catheter (Argyle™ Covidien Ltd., Dublin,  
101 Ireland) was inserted about 1 h before the beginning of sampling. No sedation was  
102 performed before inserting the intravenous catheter and during sampling. Head restraint  
103 by either a stanchion or a halter was not used, except during insertion of the intravenous  
104 catheter. The bulls were given access to water and hay at every 2 to 3 h during  
105 collection of the samples. Blood samples were collected into heparinized tubes and  
106 immediately placed in ice before centrifuging ( $1700 \times g$  for 15 min at  $4^{\circ}\text{C}$ ). The plasma

107 was decanted and stored ( $-30^{\circ}\text{C}$ ) until assay.

108

### 109 *2.3. Experiment 2*

110 A single injection of GnRH analogue (fertirelin acetate; Conceral<sup>R</sup>, Intervet,  
111 Tokyo) was given im at a dose of  $0.5\ \mu\text{g}/\text{kg}$  ( $n=6$ ). The same beef bulls that were used in  
112 experiment 1 were used for experiment 2, which took place at least 1 wk after  
113 completion of experiment 1. The blood samples for assaying INSL3, LH and  
114 testosterone were collected at  $-0.5\ \text{h}$ ,  $0\ \text{h}$ ,  $1\ \text{h}$ ,  $2\ \text{h}$ ,  $3\ \text{h}$ ,  $4\ \text{h}$ ,  $5\ \text{h}$ , and  $6\ \text{h}$  after treatment.  
115 The treatment was given immediate after the  $0\ \text{h}$  sample was drawn. Thus, blood  
116 sampling taken at  $-0.5\ \text{h}$  and  $0\ \text{h}$  are pre-treatment samples. Blood samples were  
117 collected into heparinized vacutainers by jugular venipuncture and processed as above  
118 mentioned in experiment 1.

119

### 120 *2.4. Experiment 3*

121 Six beef bulls that were used for experiments 1 and 2 were also used for  
122 experiment 3. This experiment was conducted about 6 mo after completion of  
123 experiment 2. A single dose of hCG ( $5\ \text{IU}/\text{kg}$ , im; Veterinary Puberogen<sup>R</sup>, Novartis  
124 Animal Health, Tokyo) was administered. Two pre-treatment blood samples were taken



125 at -0.5 h and immediate before the hCG treatment (0 h). The sampling was then  
126 continued at 2 h, 4 h, 8 h, day 1, day 2, day 4, day 8 and day 12 of the post-treatment.  
127 Blood collection and processing of plasma were done as mentioned above in experiment  
128 2.

129

## 130 2.5. *Hormone assays*

### 131 2.5.1. *INSL3 and testosterone*

132 Plasma concentrations of INSL3 were measured using an  
133 enzyme-immunoassay (EIA). A homologous bovine plasma EIA developed and  
134 validated in our laboratory [13] was used with a minor variation using a biotinylated  
135 canine INSL3 instead of biotinylated bovine INSL3. An anti-bovine INSL3 mouse  
136 monoclonal antibody (2-8F) and synthetic bovine INSL3 [23] were used. The minimum  
137 detection limit of the INSL3 EIA was 0.31 ng/mL, and the detection was reliable in the  
138 range from 0.31 to 20 ng/mL. The intra- and inter-assay CVs were 7.5% and 13.7%,  
139 respectively. Plasma testosterone concentrations were determined by an EIA using the  
140 procedure previously described by us [13]. An anti-testosterone rabbit polyclonal  
141 antibody and horseradish peroxidase (HRP) -labeled testosterone (Cosmo Bio Co., Ltd.,  
142 Tokyo) were used. The minimum detection limit was 0.07 ng/mL, and the reliable

143 detection range for testosterone EIA was 0.07 to 20 ng/mL. The intra- and inter-assay  
144 CVs were 6.6% and 11.3%, respectively.

145

#### 146 2.5.2. LH

147 An EIA procedure described below was used to measure LH concentrations in  
148 the bovine plasma. Eight-well strips (Corning Inc. Life Sciences, Lowell, MA, USA)  
149 were coated with 100  $\mu$ L per well of anti-rabbit IgG mouse polyclonal antibody (MP  
150 Biochemicals, Solon, OH, USA; 5  $\mu$ g/mL in 0.05 M sodium bicarbonate, pH 9.7) for 2  
151 h at room temperature. The wells were then drained and washed three times with 300  
152  $\mu$ L of 0.15 M sodium chloride. Next, 200  $\mu$ L of assay buffer (0.01M PBS, pH 7.4)  
153 supplemented with 2% bovine serum albumin (BSA; Cohn Fraction V, Sigma-Aldrich,  
154 St. Louis, MO), and 0.02% ProClin 950 (Sigma-Aldrich) was added and kept overnight  
155 at 4°C to block areas of the well that were not coated with antibody. Various  
156 concentrations of bovine LH standards (AFP11743B, NIDDK, USA; 0.31 to 40 ng/mL)  
157 were diluted with assay buffer. The plasma was centrifuged at 15,000  $\times$  g for 5 min at  
158 4°C to sediment fibrin and other particles and then supernatant was collected and  
159 diluted 2- times with assay buffer. Immediately before the assay, the wells were drained,  
160 and 50  $\mu$ L of standards or plasma samples followed by 50  $\mu$ L of the anti-bovine LH

161 antibody (Immunodiagnostik AG, Bensheim, Germany; 1: 50,000 dilution in assay  
162 buffer) were added and incubated for 2 h while shaking (180 rpm). Thereafter, 50  $\mu$ L of  
163 the biotinylated bovine LH was added (1: 50,000 dilution in assay buffer) and incubated  
164 for 1 h. The LH (AFP11743B) was biotinylated with EZ-Link NHS-PEG<sub>4</sub>-Biotin  
165 (Thermo Fisher Scientific, Waltham, MA USA). After the reaction, the wells were  
166 drained and washed three times with 300  $\mu$ L of washing buffer (0.15 M sodium chloride  
167 containing 0.05% Tween 20). Then, 100  $\mu$ L of the HRP-labeled streptavidin (KPL,  
168 Gaithersburg, MD; 100 ng/mL in assay buffer) was added to the wells and incubated for  
169 30 min. The wells were then again washed three times with saline containing 0.05%  
170 Tween 20 and incubated for another 30 min at room temperature with 100  $\mu$ L substrate  
171 solution containing 3,3',5,5'-tetramethylbenzidine (TMB; St. Louis, MO, USA). The  
172 reaction was stopped by adding 100  $\mu$ L of 2 M sulfuric acid, and the optical density was  
173 measured at 450 nm using an xMark microplate absorbance spectrophotometer  
174 (Bio-Rad Laboratories). The assay detection range was from 0.31 to 40 ng/mL. The  
175 intra- and inter-assay coefficients of variation were 4.0% and 10.7%, respectively.

176

## 177 2.6. Data analyses

178 Pulses of LH, INSL3 and testosterone concentrations in plasma samples at

179 15-min intervals during 8 h were detected with Pulse XP software kindly provided by  
180 Prof. Michael L. Johnson, University of Virginia [24]. Basal concentrations of INSL3  
181 and testosterone pulses were also determined with the Pulse XP software. Pre-treatment  
182 values at time 0 h were included in the data analysis while data of -0.5 h were excluded.  
183 The increasing rate (peak/basal concentration) of INSL3 and testosterone pulses was  
184 calculated from 15-min interval sampling. In addition, the increasing rate  
185 (maximum/pre-treatment concentration) of INSL3 and testosterone concentrations after  
186 administration of GnRH and hCG was calculated. Evaluation of LH, INSL3 and  
187 testosterone data were performed by a two-way analysis of variance (ANOVA) using  
188 the Generalized Estimating Equations (GEE) procedure of SPSS version 22 software  
189 (IBM, Somers, NY) to assess the effects of GnRH and hCG treatments. Differences in  
190 hormone concentrations were compared using pairwise comparisons of the GEE  
191 procedure by the least significant difference (LSD) post hoc test. Data are expressed as  
192 mean  $\pm$  SEM. Differences were considered significant at  $P < 0.05$ .

193

### 194 **3. Results**

195 *3.1. Pulsatile interrelationships among plasma concentrations of INSL3, LH and*  
196 *testosterone at 15-min interval for 8 hours*

197           We studied the secretory pattern of INSL3 and compared its secretion with LH  
198 and testosterone. Sampling was spaced in 15-min intervals over an 8 h session. Data  
199 analysis using the Pulse XP software showed that apart from the known pulsatile  
200 secretion of LH and testosterone, the secretion of INSL3 in the general circulation of  
201 beef bulls was also pulsatile. Fig. 1 shows the hormone profiles and detected LH,  
202 INSL3 and testosterone pulses for two representative beef bulls. In six beef bulls, during  
203 the 8 h period, a total of 28, 23 and 6 pulses occurred for LH, INSL3 and testosterone,  
204 respectively. Of the 23 INSL3 pulses, 16 (69.6%) pulses peaked within 1 h period after  
205 a peak of an LH pulse. Five bulls showed that testosterone levels started increasing  
206 within 30 min from a peak of an LH pulse. In case of the remaining bull (Fig. 1B), we  
207 were unable to detect the beginning of the testosterone pulse. In this case the  
208 testosterone pulse might have started before sampling and therefore we were unable to  
209 detect the LH pulse that induced the testosterone pulse. The frequency of LH, INSL3  
210 and testosterone pulses during an 8 h period was  $4.7 \pm 0.9$ ,  $3.8 \pm 0.2$  and  $1.0 \pm 0.0$ ,  
211 respectively. The mean increasing rate (peak/basal concentration) of testosterone pulses  
212 ( $12.9 \pm 2.0$  fold, n=6) was significantly higher ( $P < 0.001$ ) than those of INSL3 pulses  
213 ( $1.5 \pm 0.1$  fold, n=23).  
214

215 3.2. *Effect of GnRH treatment on LH, INSL3 and testosterone secretion*

216 A single dose of a GnRH analogue was administered to stimulate LH secretion to  
217 determine how increased plasma LH concentration facilitated the secretion of INSL3  
218 from the testicular Leydig cells. Mean plasma LH concentrations increased ( $P<0.01$ )  
219 dramatically 1 h after treatment and reached a maximum concentration at 2 h (Fig. 2A).  
220 Thereafter, the concentration slowly decreased but remained significantly high ( $P<0.01$ )  
221 up to 5 h post GnRH treatment but approached basal LH levels at 6 h.

222 Mean plasma INSL3 concentrations increased ( $P<0.01$ ) 1 h after the treatment  
223 and remained significantly high until 2 h ( $P<0.05$ ) (Fig. 2B). From 3 h to 5 h INSL3  
224 concentrations did not differ significantly when compared with the pre-treatment value.  
225 However, a significant increase ( $P<0.05$ ) of INSL3 concentrations was again observed  
226 at 6 h.

227 Mean plasma testosterone concentrations were increased ( $P<0.01$ ) at all time  
228 points when compared with the pre-treatment value. Testosterone levels rose at 1 h post  
229 treatment and remained significantly high until the end of sampling at 6 h (Fig. 2C). The  
230 mean increasing rate (maximum/pre-treatment concentration) of testosterone  
231 concentrations ( $7.6 \pm 2.2$ -fold,  $n=6$ ) after administration of GnRH analogue was higher  
232 ( $P<0.01$ ) than that of INSL3 ( $1.6 \pm 0.2$ -fold,  $n=6$ ).

233

234 *3.3. Effect of hCG treatment on INSL3 and testosterone secretion*

235 A single injection of hCG was administered to determine the effect of sustained  
236 levels of LH on INSL3 secretion. Mean plasma INSL3 and testosterone concentrations  
237 after administration of hCG are presented in Fig. 3. Plasma INSL3 concentrations  
238 increased ( $P<0.01$ ) 2 h after treatment and remained significantly high ( $P<0.05$ ) till the  
239 next sampling at 4 h. When compared to control no significant changes were observed  
240 at 8 h and 1 day after treatment. However, INSL3 concentrations again increased  
241 significantly on days 2 through 8 (day 2,  $P<0.01$ ; day 4,  $P<0.01$ ; day 8,  $P<0.05$ ),  
242 approaching pre-treatment level on day 12 (Fig. 3A).

243 A dramatic increase ( $P<0.01$ ) of mean plasma testosterone concentrations after  
244 treatment was observed from 2 h and continued till day 4. Thereafter, concentrations  
245 started to decrease but remained significantly elevated ( $P<0.01$ ) until day 8, reaching  
246 basal level on day 12 post-treatment (Fig. 3B). After administration of hCG, the mean  
247 increasing rate (maximum/pre-treatment concentration) of testosterone concentrations  
248 ( $10.4 \pm 2.2$ -fold) was higher ( $P<0.001$ ) than that of INSL3 ( $1.8 \pm 0.2$ -fold).

249

250 **4. Discussion**

251 For many species including bulls [14, 15] the pulsatile release of LH from the  
252 anterior pituitary stimulates immediate pulsatile secretion of testosterone from the  
253 testicular Leydig cells. Conversely, it has been reported that secretion of INSL3 is not  
254 acutely regulated by LH [22], but is stimulated by the long-term trophic effects of LH in  
255 men [1, 6–9]. However, the short-term secretory pattern of INSL3 and its relationship  
256 with LH with frequent blood sampling has not been reported. To the best of our  
257 knowledge, this is the first study to evaluate circulating INSL3 levels at 15-min  
258 intervals. We found that the nature of releasing INSL3 from the testicular Leydig cells  
259 into the general circulation of beef bulls is pulsatile, and a temporal relationship  
260 between LH and INSL3 secretion exists.

261 The secretion of INSL3 in the general circulation of beef bulls is pulsatile with an  
262 average pulse frequency of about 4 in an 8 h sampling session. The mean increasing rate  
263 of INSL3 pulses are much smaller than those of testosterone pulses, suggesting that  
264 INSL3 can act as a less-fluctuating marker than testosterone to evaluate the testicular  
265 Leydig cells status in bulls.

266 The frequency of testosterone pulses in the present study is in accordance with  
267 the previous reports [14, 15] in bulls. It has been reported that LH pulses precede  
268 testosterone pulses in bulls [14], which has been the case not only for testosterone



269 pulses but also for INSL3 pulses as shown in our present study. We noticed that 70% of  
270 INSL3 pulses peak within 1 h period from the peak of an LH pulse, indicating that in  
271 most cases INSL3 pulses are associated with LH pulses. The fewer number of  
272 testosterone pulses compared with LH pulses in an 8 h sampling session demonstrate  
273 that not all LH pulses are capable of generating a testosterone pulse, and therefore, there  
274 might be a minimum threshold value for an LH pulse to initiate a testosterone pulse  
275 whereas in case of INSL3 pulses, it seems that compared with testosterone pulses a  
276 comparatively lower minimum threshold value of LH pulses is required.

277       Upon treatment with GnRH, we noticed that similar to LH and testosterone,  
278 INSL3 concentrations also increased significantly within 1 h. The increasing rate  
279 (maximum/pre-treatment concentration) by GnRH stimulation is much lower for INSL3  
280 than for testosterone. A similar lower increasing rate of INSL3 pulses than testosterone  
281 pulses was observed under physiological condition in experiment 1 with 15-min  
282 intervals sampling. These results show that LH pulses precede INSL3 pulses in most  
283 cases. The significant increase of INSL3 concentrations within 1 h period of GnRH  
284 treatment in experiment 2 suggests that the INSL3 secretion is acutely regulated by LH.  
285 Administration of hCG, which has LH-activity, provides additional evidence regarding  
286 this issue. After hCG treatment, a significant increase of both testosterone and INSL3

287 levels was observed that sustained over a longer period of time. For both hormones, the  
288 concentrations increased shortly after treatment, remained high till day 8, but again the  
289 increasing rate is much smaller for INSL3 than for testosterone. Maintaining a  
290 significant higher concentration of INSL3 and testosterone for a longer period of time  
291 by hCG than GnRH treatment is probably due to the sustained longer activity of hCG  
292 [25]. Previously, a significant increase of testosterone in the general circulation of bulls  
293 has been shown after GnRH and hCG treatments [16–19]. In the present study, the  
294 simultaneous increase of INSL3 and testosterone concentrations within 1 to 2 h after  
295 those treatments, provide another new information that LH acutely regulates the  
296 secretion of INSL3 in bull plasma. This acute regulation of INSL3 by LH in bulls is the  
297 novel finding of our present study and is in difference to previous studies in men. One  
298 study showed that when men were treated with hCG and peripheral blood was taken  
299 daily for 8 days, testosterone concentrations increased after hCG treatment, but INSL3  
300 did not change [22] whereas other studies showed that hCG can increase INSL3  
301 concentrations in blood after 4 or 10 days of treatment when endogenous LH secretion  
302 was inhibited by androgen analogues or GnRH antagonist [26, 27].

303           In conclusion, the secretion of INSL3 in the general circulation of beef bulls  
304 occurs in a pulsatile manner. Endogenous and exogenous LH can stimulate INSL3

305 secretion soon after the treatment, suggesting the acute regulation of INSL3 by LH in  
306 bulls. Moreover, the increasing rate of INSL3 pulses is much smaller than those of  
307 testosterone pulses, and therefore we suggest that INSL3 can be used as a  
308 less-fluctuating marker than testosterone when testing the functions of testicular Leydig  
309 cells in bulls.

310

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315

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395

396 **Figure legends**

397

398 **Fig. 1.** Changes of plasma LH, INSL3 and testosterone concentrations in blood samples  
399 taken from two individual representative beef bulls (A: #1, B: #2). Blood samples were  
400 taken at 15-min intervals for 8 h. The peaks for INSL3 (⋯▶), LH (→), and testosterone  
401 (○) pulses were determined by the Pulse XP software.

402

403 **Fig. 2.** Plasma LH (A), INSL3 (B), and testosterone (C) concentrations in response to  
404 GnRH treatment (0.5 µg/kg) in beef bulls. Data are expressed as mean ± SEM (n=6). \*P  
405 <0.05, \*\*P <0.01 compared with the pre-treatment value of the corresponding hormone  
406 at 0 h.

407

408 **Fig. 3.** Plasma INSL3 (A), and testosterone (B) concentrations in response to hCG  
409 treatment (5 IU/kg) in beef bulls. Data are expressed as mean ± SEM (n=6). \*P <0.05,  
410 \*\*P <0.01 compared with the pre-treatment value of the corresponding hormone at 0 h.

411



Figure

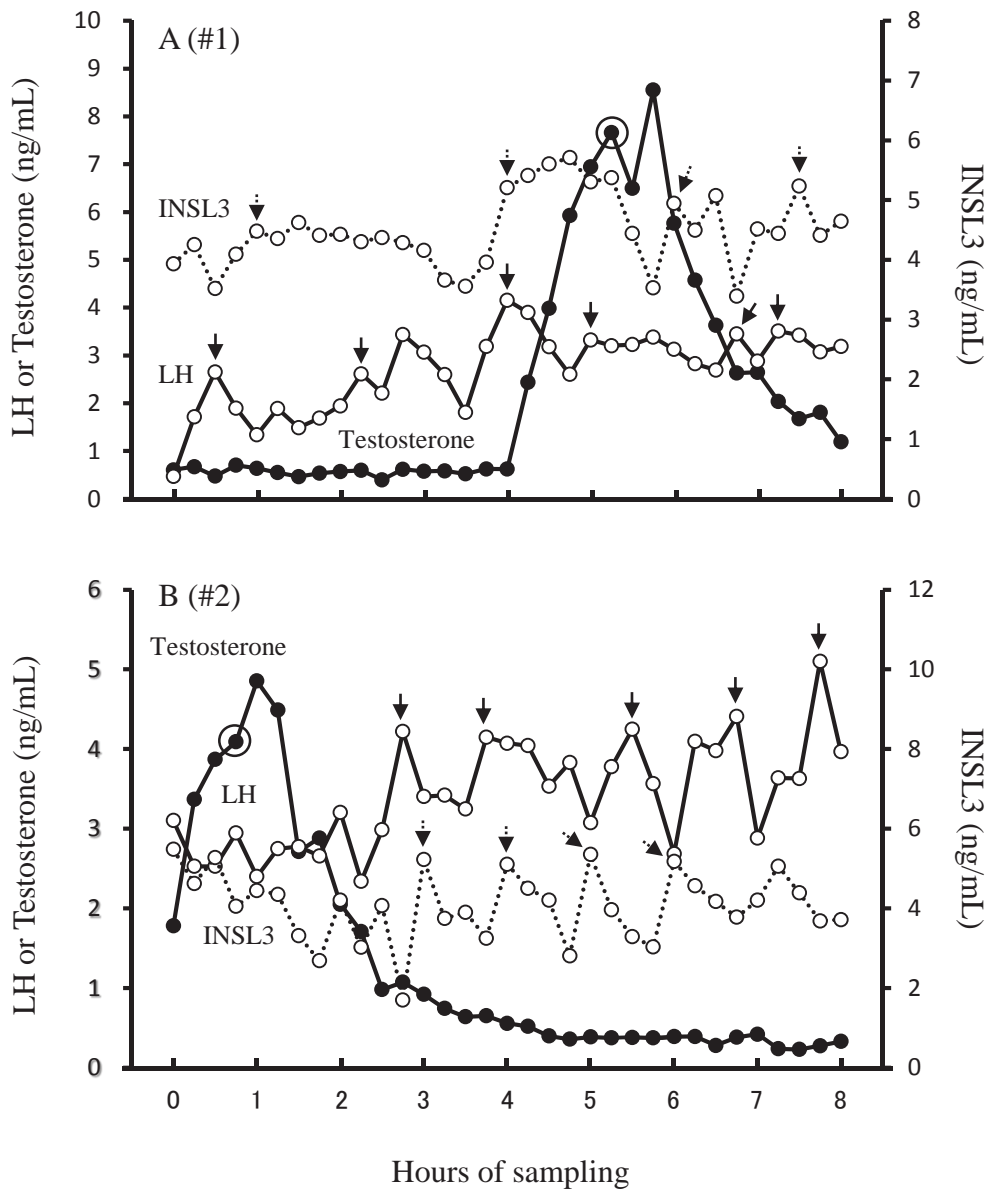
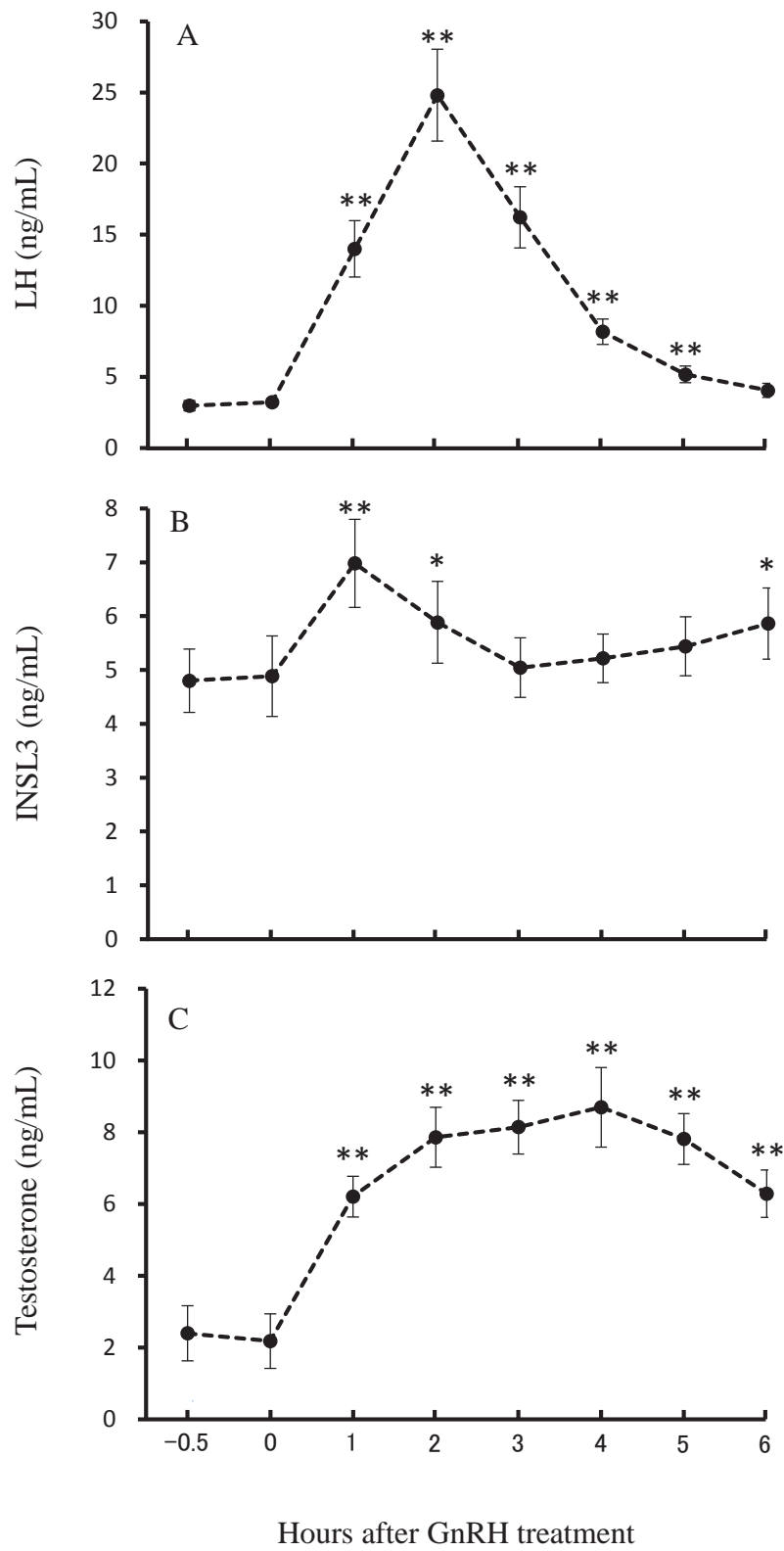
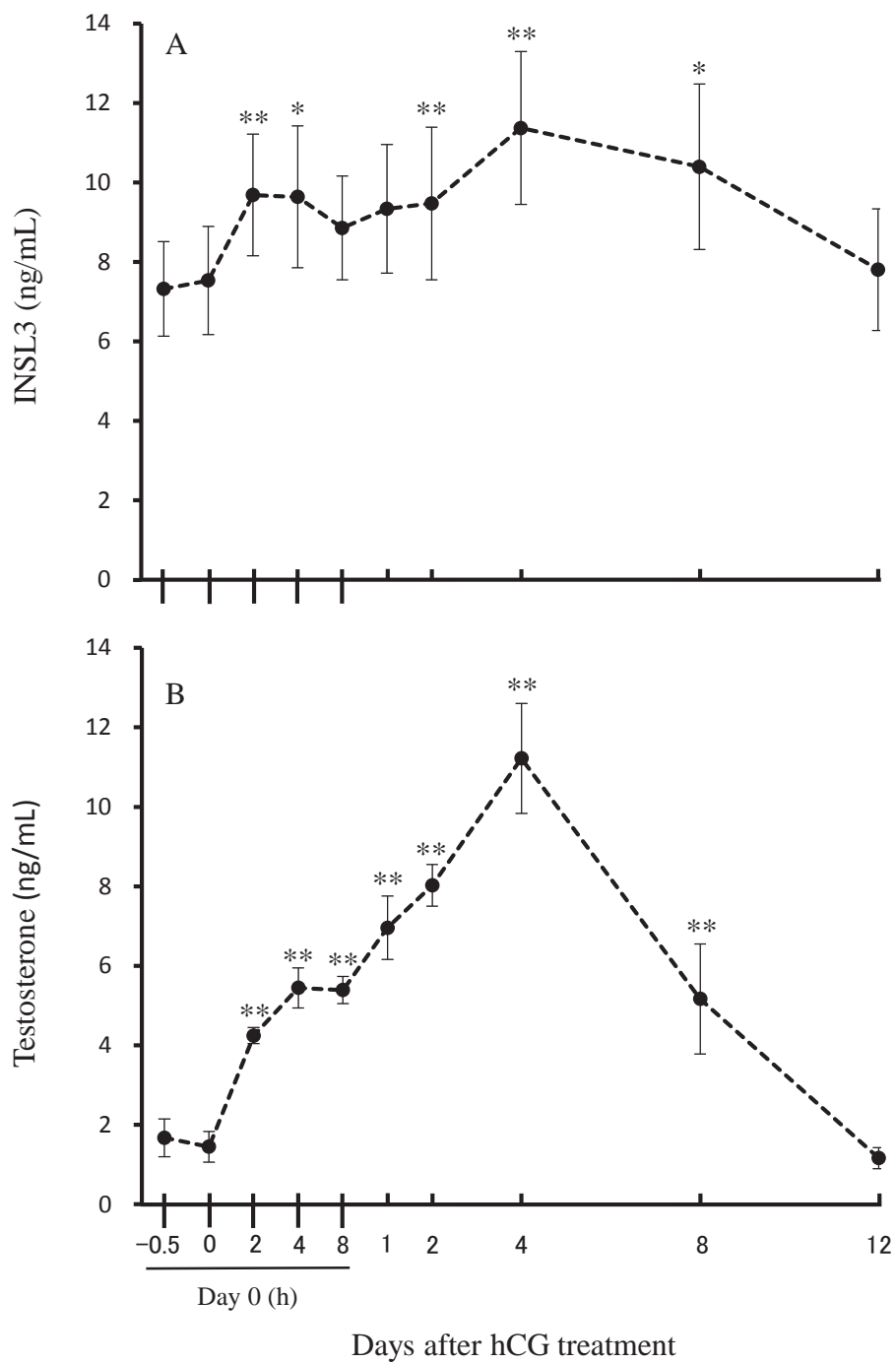


Fig. 1



**Fig. 2**



**Fig. 3**