Revised highlighted

Age related and seasonal changes of plasma concentrations of insulin-like peptide 3 and testosterone from birth to early-puberty in Thoroughbred male horses

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Abstract

The peripheral blood concentrations of insulin-like peptide 3 (INSL3) have been detected in many mammalian species, but the level of INSL3 in horse remains unknown. The objectives were to develop a time-resolved fluorescence immunoassay (TRFIA) to detect INSL3 concentrations from horse blood as well as to determine the age-related and seasonal changes of plasma concentrations of INSL3 and testosterone from birth to early-puberty in Thoroughbred male horse (n=11). Monthly blood sample and measurement of body weight, height, chest and cannon bone size were done from birth until 16 mo. The TRFIA and EIA were used to measure plasma concentrations of INSL3 and testosterone, respectively. An increase in mean body weight, height, chest and cannon bone size was observed throughout the study. The monthly blood sampling revealed an increase in mean plasma INSL3 concentrations up to 2 mo, followed by a decreasing and increasing pattern until the end of experiment at 16 mo. A high testosterone level was detected at birth followed by a sharp decrease to basal level within 1 mo, maintained low level up to10 mo before a gradual rise until 16 mo. In case of seasonality, there was no difference in mean plasma INSL3 concentrations between breeding (March to September) and non-breeding (October to February) seasons, whereas a higher ($P < 0.001$) mean plasma testosterone concentrations in the second

breeding season compared to non-breeding season was observed. In age categorized group, an increase ($P < 0.01$) in mean plasma INSL3 concentrations was noticed at pre-puberty (1 to 12 mo) and early-puberty (13 to 16 mo) compared to birth, but a lower ($P < 0.001$) mean plasma testosterone concentrations was observed at pre-puberty compared to birth and early-puberty. In conclusion, a TRFIA was developed to measure INSL3 levels in horse. An increase in plasma concentrations of INSL3 and testosterone were observed with the advancement of age, whereas for testosterone a very lower level was detected at the non-breeding season than in the second breeding season after birth in Thoroughbreed male horse. The INSL3 secretions seemed independent of seasonal influence, at least before puberty.

Keywords: INSL3; TRFIA; Breeding season; Puberty; Male horse

1. Introduction

Testicular Leydig cell has been shown as the sole secretory site of insulin-like peptide 3 (INSL3) hormone in all studied mammalian males including horse [1–3]. INSL3 is an essential factor for the trans-abdominal phase of testicular descent during the fetal period in mice [4, 5], and for preventing the germ cells apoptosis during and after puberty in rats [6] and pigs [7]. The levels of INSL3 in peripheral blood have already been reported from a wide variety of male species including humans [8–12], rats [13], cattle [14, 15], dogs [16], and goats [17]. However, the blood level of INSL3 is yet to determine in horse.

Horse is a seasonal breeder animal, which shows maximum reproductive activity during spring and summer [18]. In spring born Thoroughbred colts, the plasma concentrations of testosterone, another Leydig cell hormone, has been shown to decrease to basal level at the non-breeding season after birth followed by a dramatic increase in the second breeding season [19]. The study related to secretory pattern of INSL3 in seasonally breeding animals is limited. Only a single study in adult Roe deer, a seasonally reproducing animal, has demonstrated the changes of INSL3 at mRNA and protein level throughout a reproductive cycle [20]. The association between seasonality and INSL3 secretion is unknown in horse.

75 The age at puberty varies widely among horse breeds [21]. Both photoperiod and
76 degree of maturity play roles in the onset of puberty [22]. In colts, the onset of puberty
77 is determined by semen characteristics [23, 24] and the time when first significant
78 increase in testosterone secretion compared to baseline occurs [22, 24]. The peripheral
79 levels of INSL3 have been used as a testis-specific biomarker for the assessment of
80 pubertal development in humans [10–12], male rats [13], dogs [16], bucks [25], and
81 cattle [14]. It was reported that the dynamics of the secretory patterns of plasma INSL3
82 and testosterone during pubertal progress are different in bulls [14], male dogs [16], and
83 humans [10–12]. We speculated that being a seasonal breeder, horse may possess a
84 unique secretory pattern of INSL3 from birth until puberty.
85 The aims of the present study were: (1) to develop a time-resolved fluorescence
86 immunoassay (TRFIA) to measure INSL3 concentrations from horse blood; (2) to
87 determine the age-related changes of plasma concentrations of INSL3 and testosterone
88 from birth to early-puberty in male horse; (3) to observe the seasonal variation of
89 INSL3 secretion and its relationships with testosterone in male horse.
90
91 **2. Materials and methods**
92 *2.1. Animals*

93 Spring born colts (n = 11) from Thoroughbred broodmares raised in Hidaka
94 Training and Research Center, Japan Racing Association (JRA), Urakawa, Hokkaido,
95 Japan were used in the present study. Eight out of 11 colts were born in April, 2 in
96 March and 1 in May. Immediately after birth, testicular presence was checked manually
97 to confirm the presence of testes inside the scrotum. The colt that was missing one or
98 both testis was not included, and only the colts having both testes inside the scrotum
99 were considered for the present experiment. The selected colts had no apparent
100 abnormalities of reproductive status and remained healthy throughout the study. From 2
101 days after birth, the newborns were allowed in outdoor pasture with their dams for a few
102 hours a day and their outdoor staying was extended to 7 h from 2 weeks. Colts were fed
103 with a fistful of pellet twice daily from 2 mo of their age and they were weaned at 6 mo
104 before providing a balanced diet (JRA standards, Hiddaka, Japan). The study was
105 approved by the Animal Welfare and Ethics Committees of Obihiro University of
106 Agriculture and Veterinary Medicine.
107
108 *2.2. Blood sampling and measurement of body weight, height, chest and cannon bone*
109 Monthly sampling started at day of colts born (blood) and 1 day after birth (body
110 weight, height, chest and cannon bone measurement), and ended at 16 mo. Blood

111 samples were collected into heparinized vacutainers by jugular venipuncture between
112 13:00 and 14:00 p.m. and immediately placed in ice before centrifugation (1700 x g for
113 20 min at 4°C). The plasma was collected and immediately stored at −20°C. The
114 samples were thawed immediately before INSL3 and testosterone assays. Body weight
115 and height were measured by using a standard weighing and height measuring machine
116 for large animals, respectively. The chest and cannon bone size were measured using a
117 measuring tape. Moreover, for the purpose of INSL3 assay validation, a single blood
118 sample from 3 adult intact stallions (age, 7.0 ± 1.7 y) and 3 castrated stallions (age, 7.0
119 ± 3.0 y) was also collected.
120
121 *2.3. Preparation of testicular tissue homogenates*
122 Testicular tissue from a 1-year-old male horse was used for preparing
123 homogenates. A piece of testis tissue weighing 100 mg was homogenized in 500 µl
124 0.9% NaCL solution. The homogenates were then centrifuged at 1700 x g for 20 min at
125 4°C, and the supernatant was transferred into another tube and stored at −30°C until
126 used for checking the parallelism of INSL3 assay.
127
128 *2.4. Hormone analyses*

129 *2.4.1. INSL3 assay*

130 The plasma concentrations of INSL3 were measured by a TRFIA, which was
131 developed by minor modifications of a previously reported goat plasma INSL3-TRFIA
132 assay [25]. The DELFIA anti-mouse IgG microtitration strips coated with rabbit
133 anti-mouse antibodies (96 wells per plate; Wallac Oy, Turku, Finland) were used.
134 Immediately before the assay, colts plasma was diluted 5-fold using DELFIA assay
135 buffer (Wallac Oy, Turku, Finland). First, 50 µL of synthetic bovine INSL3 [26] for the
136 standards or 50 µL of plasma samples followed by 50 µL of anti-bovine INSL3 mouse
137 monoclonal antibody (2-8F) (1: 1,000,000 dilution in the DELFIA assay buffer, Wallac
138 Oy, Turku, Finland) [26] were dispensed in the wells and incubated for 2 h at room
139 temperature. Then, 50 µL of biotinylated canine INSL3 (2 ng/mL in DELFIA assay
140 buffer; Wallac Oy, Turku, Finland) was added and incubated for 1 h at room temperature.
141 After that, the wells were drained and washed three times with 300 µL of DELFIA wash
142 buffer (Wallac Oy, Turku, Finland). After washing, 100 µL of Eu-labeled streptavidin
143 (100 ng/mL in DELFIA assay buffer; Wallac Oy, Turku, Finland) was added to the wells
144 and incubated for 30 min at room temperature. The wells were then washed three times
145 with 300 µL of DELFIA wash buffer (Wallac Oy, Turku, Finland), and 100 µL of
146 DELFIA enhancement solution (Wallac Oy, Turku, Finland) was dispensed into each

147 well. Then, the plate was shaken at 80 rpm for 15 min at room temperature. Finally,
148 time-resolved fluorescence was measured using an ARVO MX Multilabel Counter
149 (PerkinElmer, Wallac Oy, Finland). The minimum detection limit of the INSL3-EIA was
150 0.15 ng/mL, and the detection was reliable in the range from 0.15 to 20 ng/mL. The
151 intra-assay and interassay coefficients of variation (CVs) were 7.5% and 11.5%,
152 respectively.
153
154 *2.4.2. Testosterone assay*
155 The plasma concentrations of testosterone were determined by an enzyme
156 immunoassay using a commercially available kit (ST AIA-PACK PROG II, Tosoh
157 Bioscience, Inc., San Francisco, CA, USA) according to manufacturer's instructions.
158 The minimum detectable concentration was 0.008 ng/mL, and the interassay CV was
159 14.13%.
160
161 *2.5. Data analyses*
162 We considered the period from March to September as breeding season and the
163 period from October to February as non-breeding season in spring born Thoroughbred
164 colts, according to the classification of a previous study in the same breed in same

region of the northern hemisphere [18]. The blood sampling was discontinued after 16 mo and none of the colts has any sample beyond July in the second breeding season.

The age of the colts was divided into three distinct periods: birth, pre-puberty and early-puberty. The first blood sample was collected within 6 h after foal born (birth). The distinction between pre-puberty and puberty was made based on the monthly changes in plasma testosterone concentrations. Following birth, plasma concentrations of testosterone remained very low from 1 to 12 mo of age. A significant increase in mean plasma testosterone concentrations compared to the previous month was observed between 12 and 13 mo, and the concentrations were then remained high till the end of experiment at 16 mo. Therefore, the period when testosterone remained at basal level (1 to 12 mo) was considered as pre-puberty, and from 12 mo onward when testosterone concentrations remained high considered as early-puberty (Fig. 2C).

We examined the effects of time on monthly changes in body weight, height, chest and cannon bone size as well as the plasma INSL3 and testosterone concentrations by conducting an analysis of variance (ANOVA) using the General Linear Model (GLM) procedure of SPSS ver. 25 software (IBM, Somers, NY). The effects of time on changes with the three age categories (birth, pre-puberty and early-puberty) and seasonality (breeding and non-breeding seasons) were also examined by the same

ANOVA. The differences in hormone concentrations between the birth, pre-puberty and early-puberty periods as well as between the first breeding season, non-breeding season and second breeding seasons were compared by conducting pairwise comparisons of the GLM procedure using Bonferroni as a post hoc test. The data are expressed as mean ± standard error of the mean. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Validation of an assay for measuring INSL3 in horse blood

A standard curve with the bovine INSL3 was produced in the range from 0.15 to 20 ng/mL (Fig. 1). The B/B_0 values of a serially diluted 3 y old stallion's plasma, 1 y old male foal plasma as well as testicular tissue homogenates were almost parallel to the standard curve (Fig. 1). Moreover, the plasma concentrations of INSL3 was compared between adult intact stallions and castrated stallions. The mean plasma INSL3 concentrations in intact stallions were very high (39.67 ± 4.1 ng/mL; n = 3), whereas the concentrations were very low in castrated stallion (1.93 ± 0.7 ng/mL; n = 3).

3.2. Monthly changes of body weight, height, chest and cannon bone size, and plasma concentrations of INSL3 and testosterone

201 Figure 2 illustrates the age-related changes in the mean body weight, height,
202 chest and cannon bone size as well as the plasma concentrations of INSL3 and
203 testosterone from birth to 16 mo. There was a significant effect ($P < 0.001$) of time on
204 mean body weight, height, chest, cannon bone size and the plasma concentrations of
205 INSL3 and testosterone. A gradual increase in the mean body weight, height, chest and
206 cannon bone size was observed throughout the period (Fig. 2 A and B). An increase in
207 the mean plasma INSL3 concentrations was observed up to 2 mo from birth, followed
208 by a decrease until 4 mo. The INSL3 concentrations then maintained a continual
209 increasing and decreasing pattern until the end of experiment at 16 mo (Fig. 2 C). The
210 mean plasma testosterone concentrations at birth was 0.38 ± 0.07 ng/mL. A sharp
211 decrease to approximately zero level (0.02 ± 0.00) was observed at 1 mo followed by a
212 little increase up to 3 mo before coming to very lower level again at 5 mo, maintained
213 this low level until 10 mo. The concentrations were then increased gradually until the
214 end of study (Fig. 2 C).
215
216 *3.3. Changes of plasma concentrations of INSL3 and testosterone from birth to*
217 *early-puberty*
218 The changes in the mean plasma concentrations of INSL3 and testosterone from

219 birth to early-puberty are presented in Figure 4. There was a significant effect ($P < 0.01$)
220 of age on the plasma concentrations of both hormones. The mean plasma INSL3
221 concentrations increased ($P < 0.01$) from birth to pre-puberty and early-puberty, but
222 there was no difference between pre-puberty and early-puberty (Fig. 4A). A significant
223 reduction ($P < 0.001$) in mean plasma testosterone concentrations at pre-puberty
224 compared to both birth and early-puberty was observed. There was no difference in
225 mean plasma testosterone concentrations between birth and puberty (Fig. 4B).
226
227 *3.4. Seasonal changes of plasma concentrations of INSL3 and testosterone*
228 The changes in the mean plasma concentrations of INSL3 and testosterone during
229 the breeding and non-breeding seasons are presented in Figure 3. There was a
230 significant effect ($P < 0.01$) of season on the plasma concentrations of INSL3 and
231 testosterone. The mean plasma INSL3 concentrations increased significantly ($P < 0.01$)
232 from the first breeding season after birth to second breeding season, but there was no
233 difference between non-breeding season and breeding seasons (Fig. 3A).
234 The mean plasma testosterone concentrations between first breeding season and
235 non-breeding season did not differ, but a higher ($P < 0.001$) mean plasma testosterone
236 concentrations at second breeding season compared to first breeding season and

237 non-breeding season was observed (Fig. 3B).

238

239 **4. Discussion**

240 Insulin-like peptide 3 (INSL3) has been studied extensively in many domestic
241 animals [7, 14–17] and humans [8–12]. Conversely, only a single study was performed
242 to check the expression of INSL3 in equine testis [3], and there has been no other report
243 in terms of detecting blood levels of INSL3 in horse, to the best of our knowledge. Here
244 we developed a TRFIA assay to measure INSL3 concentrations from horse blood using
245 biotinylated canine INSL3 and ant-bovine INSL3 antibody.

246 In the present study, in spring born Thoroughbred colts, a high level of plasma
247 testosterone was noticed at birth followed by a sharp decrease to basal level within 1 mo,
248 and then this lower concentration maintained before a gradual rose from 10 mo onwards.
249 This secretory pattern of testosterone was in accordance with previous report in the
250 Thoroughbred [19, 22] and Warmblood [27] colts. In the present study, the two
251 consecutive months when a statistically significant increase of testosterone secretions
252 occurred were 12 and 13 mo (Fig. 2C). Thus, we assumed that the Thoroughbred colts
253 in the present study reached to puberty at 12 mo. A significant increase in mean plasma
254 testosterone concentrations compared to base line has been used as a determinant of

255 onset of puberty in colts [22, 24]; a significant increase in testosterone compared to
256 basal level in the present and a previously reported study [19, 22] occurred almost in the
257 same period. The increase of body weight, height, chest and cannon bone size with the
258 advancement of age imply a proper growth of male foals throughout the experiment,
259 and the mean body weight at puberty in the present study in spring born Thoroughbred
260 colts was similar with previous reports in the same breed [19, 22].

261 Conversely, the nature of INSL3 secretion compared with testosterone was
262 different. There was an apparent increase in mean plasma INSL3 concentrations during
263 the first 2 mo after birth followed by a continuous rise and fall, comprising few months,
264 until the end of experiment at 16 mo. In fact, there was a tendency to increase the
265 concentrations over the advancement of time, which reflects more clearly when we
266 categorized the data into different age categories (Fig. 4). In a recent report, the
267 biweekly plasma sample from 8 to 52 weeks of male Shiba goats life showed a
268 moderate increase of plasma INSL3 secretions throughout the study, whereas the
269 testosterone secretions fluctuated [25]. Another concern regarding INSL3 secretions in
270 the present study is that there was high individual variation at some points. We speculate
271 that this could be due to individual variation in total Leydig cell mass. Further studies

are needed in order to clarify the correlation between testicular size and INSL3 secretions in male horse.

When we compared the data between breeding and non-breeding seasons, a very lower level of mean plasma testosterone concentrations in the non-breeding season after birth compared to the second breeding season was observed, which is in accord with a similar study reported [19]. The reason for such reduction of testosterone concentrations in the non-breeding season was because of reduction of gonadotropins output during the winter months as a result of shorter photoperiod and low hypothalamic pituitary output [28]. However, we did not notice such reduction for INSL3 in the non-breeding season, implying that the secretion of INSL3 in horse was probably not influenced by the pituitary gonadotropin (LH), at least before puberty. During puberty, the secretory pattern of INSL3 with frequent blood sampling (15-min intervals for 8 h) has recently been studied in Japanese Black beef bulls and male Shiba goats [15, 17]. Studies in both species have showed that the secretion of INSL3 in peripheral blood during puberty occurs in a pulsatile manner and regulates by pulsatile LH secretion. In a seasonal breeding animal, Roe deer, a peak expression of INSL3 at mRNA and protein levels was observed before the onset of breeding season, followed by a lower expression at late breeding and non-breeding seasons [20]. A significantly lower LH concentration was

290 reported in the first non-breeding season after birth compared to second breeding season
291 in the Thoroughbred colts [19]. Taking these all facts into account, we expected a lower
292 INSL3 levels in the non-breeding season than that of breeding season in the present
293 study in Thoroughbred colts, but which was not the case. The authors also felt that
294 providing LH data in the present study would have helped in describing the roles of
295 gonadotropin on INSL3 secretion more clearly in male horse. Further studies with
296 frequent blood sampling during the breeding and non-breeding seasons in stallion are
297 needed to make a precise clarification of the seasonal secretory pattern of INSL3 and its
298 interrelationships with LH in male horse.

299 After we categorized the present data into three distinct groups of advancing age
300 (birth, pre-puberty and early-puberty), there was a sharp decrease in mean plasma
301 testosterone concentrations at pre-puberty compared to both birth and early-puberty,
302 with no difference between birth and early-puberty. The pre-pubertal period in the
303 present study mostly comprised the non-breeding season; therefore, the lower
304 testosterone concentrations at this period was because of lower gonadotropins output
305 during the non-breeding season [28]. And the high testosterone concentrations at birth
306 was the carryover from the intra-uterine maternal environment, because fetal gonads of
307 horse provide precursor for maternal estrogens [29, 30]. Conversely, we noticed an

increase in mean plasma INSL3 concentrations at pre-puberty and early-puberty compared to birth, which is mostly in accord with previous reports in men [10, 12], bulls [14] and male goats [25]; where increasing blood INSL3 secretions during pubertal development were shown.

In conclusion, a TRFIA was developed to measure INSL3 levels in horse plasma. An increase in mean plasma INSL3 and testosterone was observed with the advancement of age, whereas for testosterone a lower level was detected at the non-breeding season than in the second breeding season after birth in Thoroughbred male horse. The INSL3 secretions seemed independent of seasonal influence, at least before puberty. Further studies are needed in stallion in order to clarify whether breeding season has any influence on INSL3 secretion after puberty.

Acknowledgements

We are thankful to Professor Dr. Erika. E. Büllesbach of Medical University of South Carolina, Charleston, USA for kindly providing anti-bovine INSL3 mouse monoclonal antibody (2–8F).

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413

414 **Figure legends**

415

416 **Fig. 1.** Insulin-like peptide 3 (INSL3) standard curve and a serially diluted stallion (3 y
417 old) and male foal (1 y old) plasma sample, as well as testicular tissue (1 y old)
418 homogenates. Data are shown as percentage of count bound at each concentration of
419 INSL3 standard per count bound at 0 ng/mL (B/B0). The plasma sample of stallion and
420 male foal was diluted at 1, 2, 4, 8 and 16 times. Testicular tissue homogenates were
421 diluted 2, 4, 8 and 16 times, which were equal to 2.5, 1.25, 0.62 and 0.31 mg testis
422 tissue, respectively.

423

424 **Fig. 2.** Mean body weight (A), height, chest and cannon bone size (B), and mean
425 plasma concentrations of INSL3 and testosterone (C) from birth (0 mo) to 16 mo in
426 spring born Thoroughbred male horse. Data are expressed as mean ± SEM (n =11). $P <$
427 0.001 was overall age effects for body weight, height, chest and cannon bone size as
428 well as for INSL3 and testosterone.

429

430 **Fig. 3.** Mean plasma concentrations of INSL3 (A) and testosterone (B) from birth until
431 pre-puberty in spring born Thoroughbred male horse (n = 11). Monthly blood samples

were collected from 0 to 16 mo. Results are shown for birth (day of colts born [0 mo], n=11), pre-puberty (1 to 12 mo, n=132) and early-puberty (13 to 16 mo, n=44). Data are mean ± SEM. [a–b]Values without a common superscript differed significantly (P < 0.001).

Fig. 4. Mean plasma concentrations of INSL3 (A) and testosterone (B) during breeding and non-breeding seasons in spring born Thoroughbred male horse (n = 11). Monthly blood samples were collected from 0 to 16 mo. Results are shown for the first breeding season after birth (March to September, n=73), non-breeding season (October to February, n=57), and second breeding season after birth (March to July, n=57). Data are mean ± SEM. [a–b]Values without a common superscript differed significantly (P < 0.001).

Highlights

1. A time-resolved fluorescence immunoassay was developed to detect INSL3 concentrations in horse blood
2. A slow increase in plasma INSL3 concentrations was observed with the advancement of age
3. INSL3 secretions seemed independent of seasonal influence
4. Testosterone was suppressed in the first non-breeding season after birth

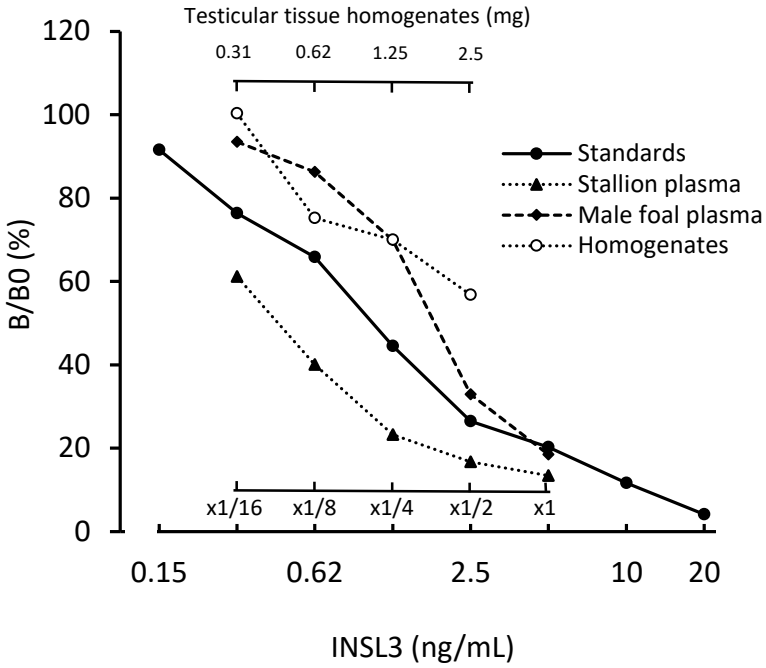
Testicular tissue homogenates (mg)
0.31
0.62
1.25
2.5
120
100
80
60
40
20
0
B/B0 (%)
Standards
Stallion plasma
Male foal plasma
Homogenates
x1/16
x1/8
x1/4
x1/2
x1
0.15
0.62
2.5
10
20
INSL3 (ng/mL)

Fig. 1

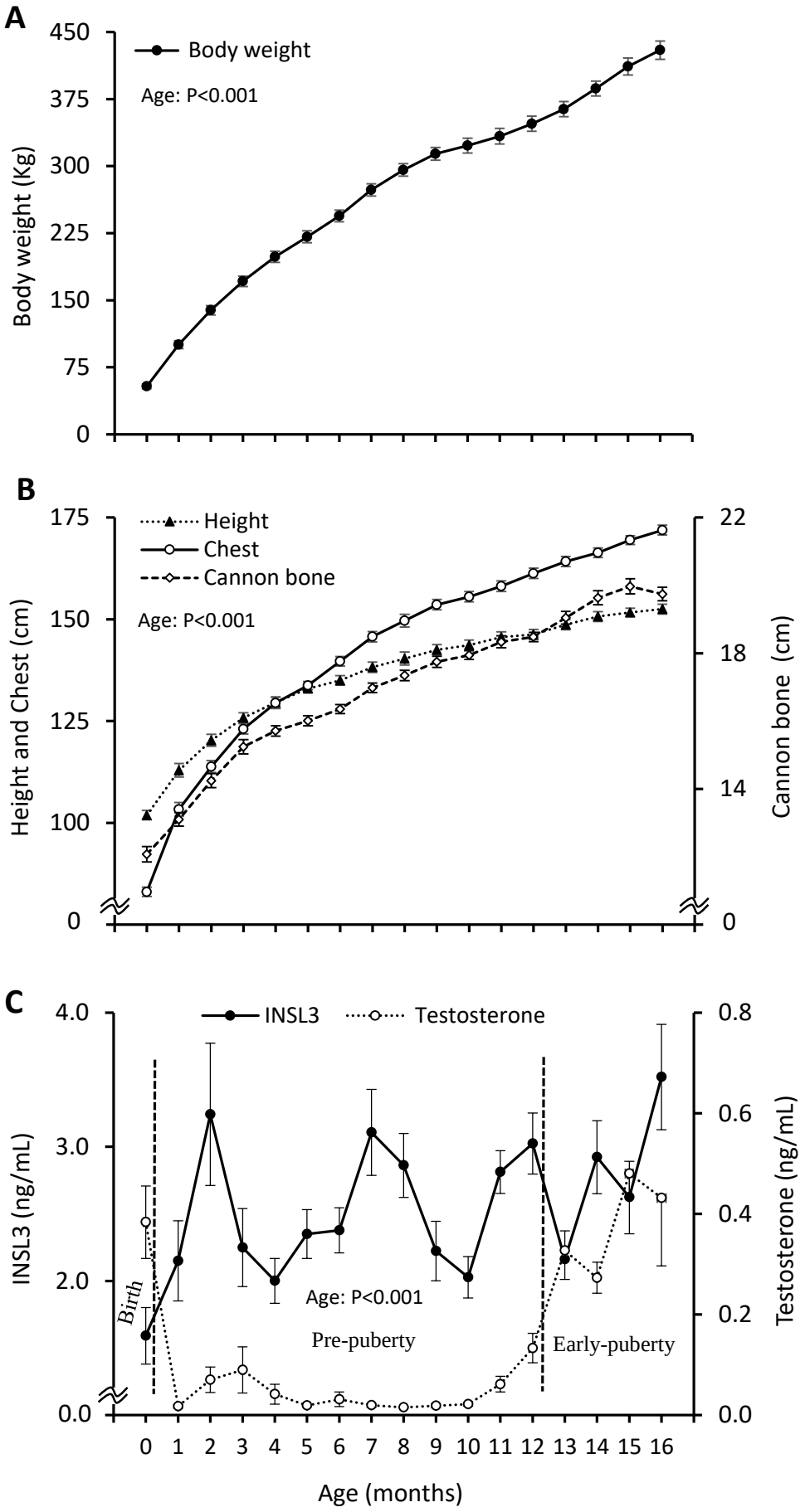
A
Body weight
Age: P<0.001
Body weight (Kg)
450
375
300
225
150
75
0
B
Height
Chest
Cannon bone
Age: P<0.001
Height and Chest (cm)
Cannon bone (cm)
175
150
125
100
0
22
18
14
0
C
INSL3
Testosterone
INSL3 (ng/mL)
Testosterone (ng/mL)
4.0
3.0
2.0
0.0
0.8
0.6
0.4
0.2
0.0
Birth
Age: P<0.001
Pre-puberty
Early-puberty
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Age (months)

Fig. 2

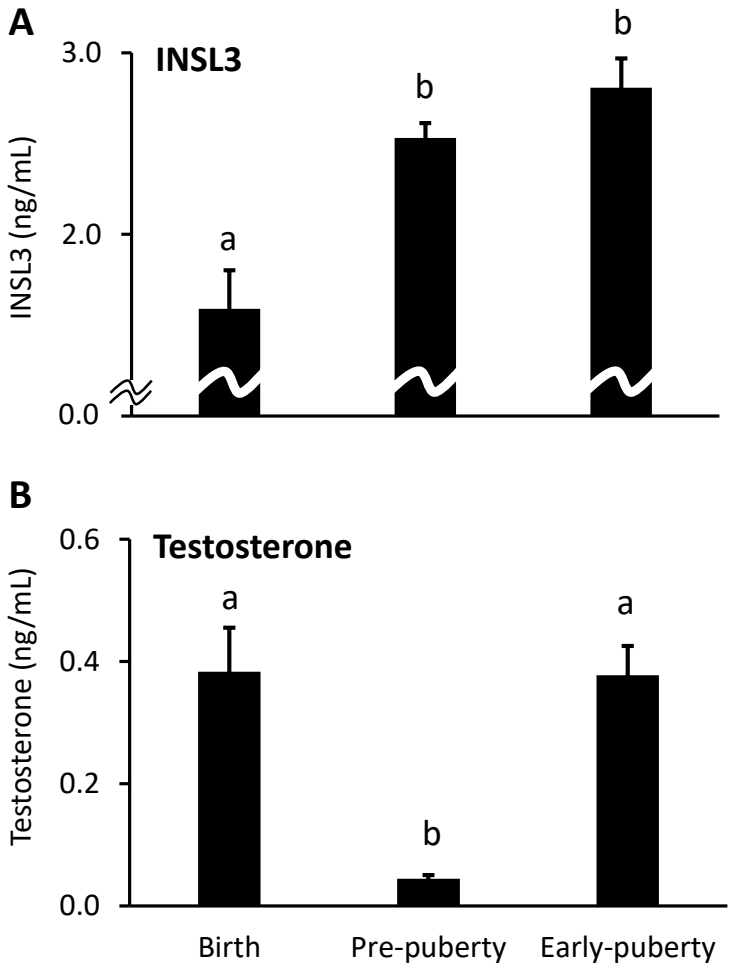
A
INSL3
3.0
2.0
0.0
INSL3 (ng/mL)
a
b
b
B
Testosterone
0.6
0.4
0.2
0.0
Testosterone (ng/mL)
a
b
a
Birth
Pre-puberty
Early-puberty

Fig. 3

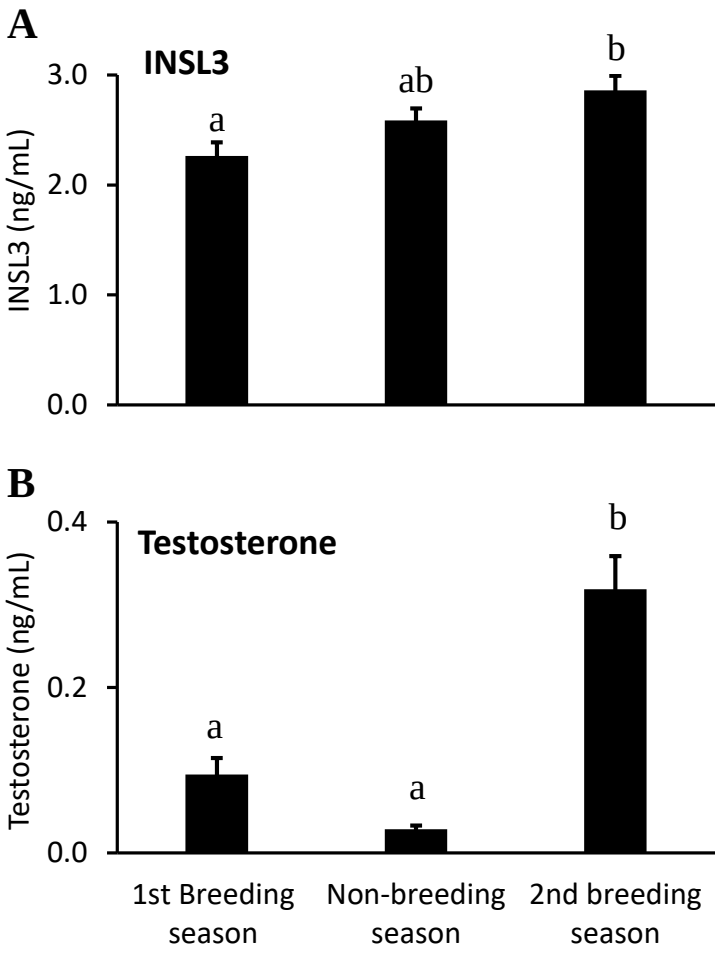
A
INSL3
3.0
2.0
1.0
0.0
INSL3 (ng/mL)
a
ab
b
B
Testosterone
0.4
0.2
0.0
Testosterone (ng/mL)
a
a
b
1st Breeding season
Non-breeding season
2nd breeding season

Fig. 4