Circulating Pituitary and Gonadal Hormones in Spring-born Thoroughbred Fillies and Colts from Birth to Puberty

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Abstract. The present study was conducted to elucidate the profile of circulating gonadotropins and gonadal hormones from birth to puberty and relationship between gonadal seasonality and hormonal secretion in both sexes of Thoroughbred horses. Spring-born colts (n=6) and fillies (n=9) were blood sampled weekly from jugular vein from birth to 60 weeks of age. Circulating FSH, LH, prolactin, testosterone, progesterone, estradiol-17 β , and immunoreactive (ir)-inhibin were measured by radioimmunoassay. In both sexes, the steroid hormones levels were remarkably high at birth, rapidly dropped within a week and remained at the lower levels until the start of second spring after birth. Ir-inhibin was also high during the birth, remaining lowest during winter and again increasing towards the second summer. There was an increase in FSH concentration in foals during the first summer months after birth and in the next summer, the FSH concentration along with that of LH increased significantly. The seasonal increase in circulating prolactin was remarkable even in the first year, and no differences were noted between the two summers. These results clearly demonstrated that the hypothalamo-pituitary axis is already responsive to changes in photoperiod and secrete prolactin similar to adult horses, but pituitary gonadotrophs for FSH and LH secretion is less sensitive. When the values of these hormones in the second breeding season after birth were compared with adult values of the respective sex in the breeding season, no significant differences were observed, indicating that spring-born fillies and colts have already attained the stage of puberty at the second breeding season after birth.

Key words: Prolactin, Puberty, Seasonality, Thoroughbred

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Equines are seasonal breeders with activity being highest as days grow longer [1]. There is no univocal reporting of the age of puberty in horses, which has been reported to vary in the range of 7.8–37 months [2]. This variation can be attributed to differences in breeds [3–5], locales, management and puberty definition [6, 7]. Furthermore, there have been few studies on Thoroughbreds [7–9], and most of them were conducted in southern hemisphere. Geographical location influences the season, and there have been fewer reports regarding puberty in horses from the northern hemisphere.

There is a dearth of information on the characteristics of circulating gonadotropins and gonadal hormones in Thoroughbred foals immediately after birth through pre-pubertal age until puberty. Previous studies provided information on relatively shorter periods [10, 11] of developmental periods after birth, and those studies focused on certain hormones [3, 4, 6] only. In seasonal breeders, there is

evidence for the involvement of both photoperiodic cues and degree of maturity in the onset of puberty [8]. The photoperiodic information is thought to be conveyed to the reproductive and prolactin axis via changes in circulating concentrations of melatonin [12]. To date, sequential profiling of prolactin has not been done in horses for a long term after birth.

Puberty is a process that results from a complex series of coordinated neuroendocrine changes leading to internal and external physical changes in primary and secondary sexual characteristics and eventual reproductive competence. Although spermatogenesis [13] continues throughout the year in stallions, the reproductive ability is optimal in the breeding seasons. Similarly, mares are seasonal in reproduction [14], the reproductive activity being highest in spring and summer months. A review on puberty of horse [2] indicates that the focus has been on a single sex with reference to specific hormones. The aim of the present study was to perform an integrated study involving both sexes (colts and fillies) with thorough consideration of major hormonal regulators of the hypothalamo-pituitary-gonadal (HPG) axis.

Despite many criteria for distinguishing puberty in animals, the criteria followed by Brown-Douglas *et al.* [8] with supporting data from comparison with adult Thoroughbred values has been adopted

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in this study. The objectives of this study were to 1) develop a profile of pituitary and gonadal hormones from birth until 60 weeks of age in spring-born Thoroughbred male and female foals, 2) determine approximate age at puberty of spring-born colts and fillies with reference to adult values in the breeding season, and 3) find out the association between seasonality and prolactin hormone in spring-born Thoroughbred colts and fillies.

Materials and Methods

Animals

Six colts and nine fillies born during spring from Thoroughbred broodmares kept at the Equine Research Institute of the Japan Racing Association (JRA), located in Utsunomiya (36° 33' 36" N), Tochigi, Japan were used in this experiment. Secondary data on daylength was procured from the website of the Japan Meteorological Agency. The subjects were born in spring, from mid April to the end of May. The newborns were allowed outdoors in a pasture with their dams from the second day after birth for a few hours a day. After 2 weeks, they were kept indoors at night and outdoors during the day for 7 h. Foals were fed with a fistful of pellet diet twice daily after they were 2 months old. They were weaned at 6 months and switched to a balanced diet (JRA standards, Hidaka, Japan). On reaching one year of age, the colts and fillies were separated and kept in a pasture. Seven fertile mares (5–9 years old) and 10 stallions (6–8 years old) were employed for the comparative study with fillies and colts respectively.

Body weight and blood sampling

Weekly blood samples were taken from colts and fillies at 1300–1400 h from birth until 60 weeks of age for the analysis of changes in circulating follicle-stimulating hormone (FSH), luteinizing hormone (LH), prolactin, immunoreactive inhibin (ir-inhibin), testosterone, progesterone, and estradiol-17 β . Weekly body weights were measured using a standard weighing machine for large animals. Blood samples were taken twice a week in mares during the estrous cycle (follicular and luteal phase) during March and April and once a month in stallions during the breeding (March–September) and non-breeding (October–February) seasons for a period of two years. Blood samples were collected from Jugular vein into heparinized vacutainers. Plasma was harvested and stored at –20 C until assayed.

Radioimmunoassay of FSH, LH, ir-inhibin, testosterone, progesterone, and prolactin

The plasma concentration of FSH and LH were determined by homologous double-antibody equine RIA methods as described previously [15]. The intra- and inter-assay coefficients of variance were 4.9% and 12.2% for FSH and 12.6% and 15.1% for LH, respectively. The concentrations progesterone, testosterone and estradiol-17 β were determined by double-antibody RIA systems using ¹²⁵I-labeled radioligands as previously described [16]. Anti-sera against progesterone (GDN 337), testosterone (GDN 250) and estradiol-17 β (GDN 244) were used in each RIA. The intra- and interassay coefficients of variance were 7.3% and 14.3% for progesterone, 6.3% and 7.2% for testosterone and 6.7% and 17.8% for estradiol-17 β , respectively. Plasma ir-inhibin concentrations were

measured using a rabbit antiserum against purified bovine inhibin (TNDH 1, Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology) and ¹²⁵I-labeled 32-kDa bovine inhibin, as previously described [17]. The results were expressed in terms of 32-kDa bovine inhibin. The intra- and interassay coefficients of variance were 8.0% and 16.2%, respectively. The plasma concentration of prolactin was measured by RIA using rat anti-sera against equine prolactin (AFP-261987) and a reference standard (AFP-8794B) as previously described [1]. The intra- and interassay coefficients of variance were 7.1% and 9.8% respectively.

Statistical analysis

The birth date ranges for the fillies and colts were 54 and 43 days respectively, and the mean birth date (May 7 for fillies and May 1 for colts) was calculated; the data were mounted over ages of 1–60 weeks. All the values were expressed as means $\pm$ SEM. Data for breeding season were calculated by taking the averages of values falling in between March 21 and September 23, and rest of the values in a year were regarded as belonging to the non-breeding season. One-way ANOVA with Tukey's multiple comparison tests was performed to detect the significant changes in the concentration of hormones from the birth until 60 weeks of age. Pearson's *r* was calculated to find correlation between variables. Values were considered significant at *P* < 0.05. All calculations were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California USA; www.graphpad.com).

Results

Body weight

The mean weight (Fig. 1a) of the colts (55.8 $\pm$ 5.1 kg) at birth was greater than that of the fillies (54.2 $\pm$ 5.7 kg). Fillies had an accelerated growth, surpassed the weight of the colts within a week (67 $\pm$ 4.4 kg vs. 64 $\pm$ 10.4 kg) and maintained the superiority throughout the rest of the age. Both sexes weighed double their birth weight at the age of 11 weeks. At the end of the study period, colts and fillies were 373.7 $\pm$ 19.2 kg and 387.8 $\pm$ 18.2 kg, respectively. The weekly gain of body weight (Fig. 1b) showed a decreasing tendency with the increase in age in both sexes. Colts and fillies showed a significant negative nonlinear trend (slope = –0.042 and –0.041 respectively, *P* < 0.0001) in the body weight gain per week with advancing age. There was no statistical significance for the difference in weights between colts and fillies at any point of age. Fillies attained puberty by 52 weeks of age, at which time they weighed 352.5 $\pm$ 4.9 kg, and colts attained puberty at 54 weeks, at which time they weighed 348.7 $\pm$ 5.5 kg.

LH, FSH and ir-inhibin

Filly: LH (Fig. 2a) remained at its basal level after the birth and started rising after 45 weeks of age. By 53 weeks of age, the value (2.39 $\pm$ 1.27 ng/ml) was significantly higher than at the birth (0.27 $\pm$ 0.12 ng/ml). LH was at its maximum (4.32 $\pm$ 0.97 ng/ml) when fillies reached the age of 58 weeks. There was no significant difference in the mean LH value in the second breeding season when compared with the luteal phase LH of mares (Fig. 3a). The FSH (Fig. 2b) value of the fillies was at the basal level (1.21 $\pm$ 0.15

Fig. 1. Weekly means $\pm$ SEM a) body weights and b) body weight gain and their nonlinear regression (light color) in colts and fillies.

ng/ml) at birth, which increased but nonsignificantly after 6 weeks, and reached 3.28 ± 1.70 ng/ml at 13 weeks of age and then slowly decreased towards the non-breeding season. In the next breeding season at 48 weeks of age, the value (5.10 ± 1.16 ng/ml) increased significantly, continued to increase and reached the maximum value (7.53 ± 1.29 ng/ml) at 58 weeks; there was no significant difference in the mean FSH value in the second breeding season when compared with FSH in mare in the breeding season (Fig. 3b). The fillies were born with a high amount (2.69 ± 0.37 ng/ml) of ir-inhibin (Fig. 2f), which dropped significantly to 1.82 ± 0.31 ng/ml in the following week and continued to decrease towards winter, reaching the lowest value (0.49 ± 0.04 ng/ml) at 30 weeks of age. Increasing values were observed towards the summer months, and maximum value, 1.10 ± 0.16 was reached at 54 weeks of age in the second breeding season, the average for which was equal to that of mare's ir-inhibin value (Fig. 3f).

Colt: There was a nonsignificant increase in LH (Fig. 4a) at 7 weeks (0.31 ± 0.12 ng/ml) after birth (0.15 ± 0.04 ng/ml), which declined towards the non-breeding season and reached its nadir (0.07 ± 0.01 ng/ml) in February at 34 weeks of age. The value started to increase in spring and reached the significantly highest point (0.57 ± 0.21 ng/ml) at 50 weeks of age. During birth, the FSH level (Fig. 4b) was 1.18 ± 0.32 ng/ml, and it increased nonsignificantly, reached the maximum value (1.87 ± 0.19 ng/ml) at 11 weeks of age and then dropped towards the fall months. Beginning at 35 weeks of age in winter, FSH started to increase and reached a significantly high concentration (3.99 ± 1.00 ng/ml) at 46 weeks of age in spring. It was at its maximum (4.40 ± 1.82 ng/ml) at 52 weeks and started to drop towards the end of breeding season. Colts were also born with a high concentration (1.49 ± 0.29 ng/ml) of ir-inhibin (Fig. 4f) that slowly decreased towards the significantly lowest value (0.54 ± 0.07 ng/ml) in fall at 21 weeks of age. Its nadir, 0.52 ± 0.04 ng/ml, was observed at 32 weeks in winter, and the value rose gradually

towards the point of significance (1.34 ± 0.32 ng/ml) at 56 weeks of age in the next summer and then reached the maximum value, 1.52 ± 0.38 ng/ml, by the end of study period. The average concentrations of LH, FSH and ir-inhibin were not significantly different between colts in the second breeding season and stallions in breeding season (Fig. 5a, 5b, and 5f).

Progesterone, estradiol-17 β and testosterone

Filly: Fillies were born with a high plasma concentration (5.52 ± 1.77 ng/ml) of progesterone (Fig. 2d) that dropped significantly (1.20 ± 0.79 ng/ml) in the following week and remained at the basal level (below 0.3 ng/ml) from 3 to 47 weeks of age. The value started to increase at 48 weeks (0.97 ± 0.76 ng/ml) of age in the next spring, and significantly exceeded the non-breeding season values at the age of 52 weeks (4.10 ± 1.07 ng/ml) and maintained dominance thereafter. Their average value in the second breeding season approached the progesterone levels of mares in the luteal phase (Fig. 3d). The plasma concentration of estradiol-17 β (Fig. 2e) was also significantly high (153.99 ± 20.12 pg/ml) at birth then dropped significantly in the second week (58.87 ± 15.10 pg/ml). The value slowly dropped towards the mid winter and reached its nadir, 9.62 ± 1.47 pg/ml, at 33 weeks. Beginning at 47 weeks of age in spring, it started to increase (45.74 ± 11.22 pg/ml) and reached a peak of 76.29 ± 33.10 pg/ml at 50 weeks. The plasma concentration of estradiol-17 β in fillies in the second breeding season was similar to the mare's level in the follicular phase (Fig. 3e).

Colt: Colts were born with a high plasma concentration (619.10 ± 152.48 pg/ml) of testosterone (Fig. 4d) that significantly fell to 90.23 ± 13.28 pg/ml in the second week and then remained at the baseline prior to 47 weeks (305.31 ± 38.12 pg/ml) of age in spring, at which point the value was significantly higher than the baseline values. The maximum concentration, 578.91 ± 150.55 pg/ml, was observed at 54 weeks of age. The plasma concentration of estradiol-17 β

Fig. 2. Weekly hormonal profile of a) LH, b) FSH, c) prolactin, d) progesterone, e) estradiol-17 β and f) ir-inhibin from birth to 60 weeks of age in fillies. Note the correlation between day length (light color) and prolactin in Fig. c. The shaded portions represent the natural breeding seasons. Results are expressed as means $\pm$ SEM of 9 animals.

(Fig. 4e) varied greatly among the colts. It was high at birth, with a range of 48.19–291.03 pg/ml, and then decreased in all individuals towards the winter months. In one colt in particular, the first burst in estradiol-17 β (595.95 pg/ml) was noticed at 43 weeks, and the value increased further and peaked (776.13 pg/ml) at 52 weeks of age. There were no significant differences in the plasma concentrations of testosterone (Fig. 5d) and estradiol-17 β (Fig. 5e) between colts in the second breeding season and stallions in the breeding season.

Prolactin

Fillies and colts had a low prolactin concentration in circulation at birth, the value being 2.82 ± 0.26 (Fig. 2c) and 3.68 ± 0.27 (Fig.

4c) ng/ml respectively. It started to increase towards the summer months after birth and reached a significantly high value of 5.93 ± 0.63 ng/ml for fillies at 12 weeks and 8.44 ± 1.87 ng/ml for colts at 11 weeks. These values started to decrease towards a minimum of 3.28 ± 0.33 ng/ml at 38 weeks for fillies and 3.95 ± 0.34 ng/ml at 39 weeks for colts. With the advance of spring, the concentration rose again and significantly differed at 57 and 58 weeks for fillies (6.13 ± 0.45 ng/ml) and colts (9.85 ± 2.20 ng/ml) respectively, when compared with the winter minimal values. The average concentrations of prolactin in both breeding seasons for fillies (Fig. 3c) and colts (Fig. 5c) were the same as the levels of mare and stallion (Figs. 3c and 5c) respectively in the breeding season. The plasma concentration

Fig. 3. Average ($\pm$ SEM) concentrations of a) LH, b) FSH, c) prolactin, d) progesterone, e) estradiol-17 β and f) ir-inhibin during the breeding season at birth (BB), non-breeding season (NB) and second breeding season after birth (SB) of fillies (n=9) and comparison of those values with the respective hormone levels in mares (n=7) at the follicular phase (FP) and luteal phase (LP) during breeding season. Significant differences in one-way ANOVA are denoted by * ($P<0.05$), ** ($P<0.001$) and *** ($P<0.0001$).

of prolactin over the weeks was positively correlated with the day length (Figs. 2c and 4c) of those weeks, with the Pearson correlation coefficient (r) being 0.54 and 0.64 for fillies and colts respectively.

Discussion

Puberty is associated with increased secretion of gonadotropins, secretion of testosterone, estradiol-17 β , and progesterone in response to discharge of LH and the start of spermatogenesis [18] and ovulation. It is a precisely coordinated phenomenon of the hypothalamo-pituitary-gonadal (HPG) axis. Thus, this study takes into account of all the major hormones involved in HPG axis function of both the colts and fillies. Hereafter unless otherwise specified, hormonal descriptions apply to both sexes. There was a small increase in FSH

(Figs. 2b and 4b) in the breeding season of birth, with peaks during third month of life. A post-natal increment in gonadotropins is evident in other species [19, 20] and reflects the early follicular increment and progression of testicular maturation respectively, which perhaps is responsible for sexual maturity after puberty. Although there was individual variation, the plasma LH concentration increased slightly after 2 weeks of birth in colts. The plasma concentration of FSH reached significantly higher levels earlier than LH in both sexes in the next breeding season, at which time the animals exceeded 46 weeks of age. Circulating ir-inhibin, the concentration of which was high at birth, slowly decreased with the advance of age of the foals. Equine fetal gonads undergo remarkable enlargement and regression during pregnancy and secrete high amounts of inhibin [21, 22]. This decline in circulating ir-inhibin can be explained by the decreasing

Fig. 4. Weekly hormonal profile of a) LH, b) FSH, c) prolactin, d) testosterone, e) estradiol-17 β , and f) ir-inhibin from birth to 60 weeks of age in colts. Note the correlation between day length (light color) and prolactin in Fig. c. The shaded portions represent the natural breeding seasons. Results are expressed as means $\pm$ SEM of 6 animals.

secretion by the regressing gonads after birth with further suppression by the early FSH increase in foals. In the next breeding season, after they reached the age of 56 weeks, the plasma levels of ir-inhibin increased significantly in the colts. Inhibin has been implicated as a marker of testicular function of stallions [23–25], and a gradual increase in circulating inhibin is shown during the follicular phase in mares [26–28].

Both colts and fillies were born with a high amount of steroid hormones that dropped abruptly within 24 hours [10, 11]. The fetal gonads of horse provide precursors for maternal estrogens [21, 22], and the high concentration at birth is the carryover from the intrauterine maternal environment and subsequent metabolism after birth. During

the non-breeding season, all steroids were at the basal level, and in next breeding season after 47 weeks of age, remarkable increments were observed. The reduction in gonadotropins and in turn steroids in the non-breeding winter months could be due to the modulatory role of decreased photoperiod and low hypothalamic GnRH output [29]. The HPG axis is regulated by the negative feedback of steroids on gonadotropins [7], which is difficult to appreciate in this study due to the long interval sampling procedure. However, colts and fillies in this study exhibited a clear increase in gonadotropins and concomitant gonadal steroid hormones during the second breeding season, which indicates that the animals reached puberty.

Considering puberty as a point when progesterone levels exceed

Fig. 5. Average ($\pm$ SEM) concentrations of a) LH, b) FSH, c) prolactin, d) testosterone, e) estradiol-17 β and f) ir-inhibin in the breeding season at birth (BB), non-breeding season (NB) and second breeding season after birth (SB) of colts ($n=6$) and comparison of those values with the respective hormone levels in stallions ($n=10$) during the breeding season (StBS) and non-breeding season (StNS). Significant differences in one-way ANOVA are denoted by ** ($P<0.001$) and *** ($P<0.0001$).

2 ng/ml [8] and rise by 2 standard deviation (SD) above the average value, the fillies reached puberty by 52 weeks of age, at which point they weighed 352.4 ± 4.9 kg. Similarly, when an excess of 500 pg/ml [6] and a 2 SD increment over the average value of testosterone is taken as an indication of pubertal age, colts attained puberty at the age of 54 weeks, at which point they weighed 348.7 ± 5.5 kg. Colts and fillies had a prepubertal rise in gonadotropins, which is much earlier than the steroid peaks, and such gonadotropin rise has been shown to be a result of increase in pulse frequency in other domestic animals [18, 30]. Similar phenomenon can be assumed in the case of colts and fillies during the prepubertal period. An earlier study in the southern hemisphere [8] revealed that autumn-born foals reach puberty earlier than spring-born ones but body weight was higher in spring-born foals at puberty. The spring-born foals in

that study reached puberty at an age and weight similar to those of the spring-born foals in the present study. When all the hormones concentrations of the colts and fillies in the second breeding season after birth were compared with the respective hormone levels in the stallions and mares in the breeding season, there were no significant differences. This provides additional support to our conclusion that the colts and fillies reached puberty by 60 weeks of age.

The circulating prolactin concentration was episodic in both colts and fillies, the crests being during June-August which is the transition of spring to summer in the northern hemisphere. The trough of the prolactin episode was observed during the months of December-February, which is the nadir of the winter season. In our experiments, prolactin in both sexes correlated positively with the day length. In fact, an earlier study [1] in stallions and

geldings showed that the correlation is stronger with the daylength than with temperature. It is a known fact that melatonin secretion is lower during increasing photo periods. The prolactin axis of the mare is negatively regulated by melatonin during the spring and early summer months [12]. Notably, the prolactin concentrations were similar between the immediate breeding season at birth and the second breeding season after the birth. This phenomenon was identical in both the colts and fillies. The maturity of animals did not appear to influence the prolactin concentration. Prolactin has been demonstrated to be involved in steroidogenesis [31] in humans and immunization against estrogens did not affect prolactin levels in stallions [32]. Steroid hormones in colts and fillies did not correlate with prolactin in this study also. This is further supported by the result that there is a gonadal-independent effect of season on lactotroph population [33] and an absence of prolactin receptors on gonadotrophs cells [34]. The prolactin hormone profiles of the colts and fillies in this study reflected seasonality without any direct possibility of modulation of other hormones, at least in the breeding season of birth as there was no overall significant correlation of prolactin with gonadal hormones and gonadotropins. The prolactin action on gonadotrophs during the breeding season has recently been shown to be tightly regulated by dopamine [29]. Profiling of dopamine can thus give better understanding of the relationship between prolactin and other hormones. Mares discharge prolactin from follicular fluid into the peritoneal cavity during ovulation, although it may not contribute much to the plasma levels [35]. In general, the increase in prolactin in the breeding season of birth in this study can be taken as an indication of pituitary maturity, especially of the lactotroph in these animals, as the levels are similar to those in the second breeding season which in turn equaled the adult values in the breeding season (Figs. 3 and 5).

In summary this study clarifies the profiles of reproductive hormones secreted from pituitary and gonads of spring-born Thoroughbred colts and fillies from birth until puberty. These spring-born (breeding season) animals had a high amount of steroid hormones that dropped within a week to the nadir, and all hormones measured in this study reached the maximum levels in the next breeding season after birth. Spring-born fillies and colts reached puberty in the next breeding season after birth, and before the age of 60 weeks, when all hormones concentrations and body weight approached the respective adult Thoroughbreds' values. Seasonal high prolactin in young and pubertal horses during the breeding months of the year is independent of the maturity of the animals and is positively correlated with the day length.

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