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Profiles of oestradiol, testosterone and androstenedione in stable *vs.* forest born young Konik Polski horses*

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Blood plasma profiles of equine sex steroids are useful in diagnose and for different scientific purposes. The aim of this study was to monitor oestradiol, testosterone and androstenedione blood plasma profiles in foals of age between 6 and 18 months, in order to establish the reference levels for male and female of young Konik Polski horses. The effects of birth place (stable *vs*. forest), age and season. Fifty two Konik Polski horses, born in the forest reserve (n=26) and in the stable (n=27) were studied.

Level of oestradiol differed between sexes at the age of 12 months, whilst androgens were higher in males of every age group. At all ages the level of oestradiol, testosterone and androstenedione did not differ between stable and forest born colts and fillies, except for fillies at 18 months of age. The highest level of oestradiol in both colts and fillies was found at the age of 15 months, *i.e.* at the late summer. In colts, testosterone and androstenedione concentrations were at their nadir at 6 and 9 months of age, then they rose abruptly at month 12 of age and remained significantly elevated until month 18. In fillies, the highest concentrations of these androgens were found in August-September, when the females were 15 months old. The profiles and the role of sex steroid hormones in social and reproductive behaviours are discussed.

KEY WORDS: androstenedione / colts / fillies / Konik Polski horses / oestradiol / testosterone

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Knowledge about the plasma profiles of sex steroids in horses is important for diagnosticians to assess physiological and pathological reproductive mechanisms. It is also vital as a reference for different scientific purposes. In available literature information on the range of oestradiol, testosterone and androstenedione in prepubertal and mature horses differs according to the source [Kosiniak and Bittmar 1987, Roser and Hughes 1992, Nagata *et al*. 1998, Stewart and Roser 1998, Lemazurier *et al*. 2002, Medan *et al.* 2004, Amer *et al.* 2007, Opałka *et al.* 2010, Haffner *et al.* 2010]. Determination of the function of sex glands in an adult horse is routinely done by ultrasonic examination and evaluation of sex steroids levels. In foals and very young colts and fillies, where transrectal examination could be more difficult, the reference profile of steroid hormones seems to be very useful. However, it has been suggested that horses of more primitive breeds may differ in the level of sex steroids to other horse breeds [Haffner *et al.* 2010]. In the latter study, discrepancies considering relatively low testosterone concentrations in stallions as compared to hitherto studies on other equine breeds were revealed.

Konik Polski horse is the Polish native horse that is believed to be a descendant of the extinct wild Tarpan horse. Horses of this breed are mainly kept under standard stable conditions, but large numbers of them are maintained in free-roaming familial groups within forest reserves. The forest breeding stock of Koniks kept in Popielno Station of Ecological Agriculture and Preservation Animal Breeding differs to some extent genetically from the stabled stock, since no stable-born horses have been introduced to the forest. In the study on Popielno Konik stallions [Opałka *et al.* 2010], faecal androgen profiles differed between males kept for reproduction in the stable and those in the forest reserve. It is, however, not known wheather these differences could be linked to genetic or environmental effects.

Since horses are long-day breeders, the effect of the seasons is an important factor in their reproduction [Ginther 1992, Roser and Hughes 1992, Nagata *et al.* 1998, Stewart and Roser 1998, Lemazurier *et al.* 2002]. It is well known, that photoperiod also regulates the endocrinology of the maturing foals [Nogueira *et al*. 1996, Lemazurier *et al.* 2002b, Brown-Douglas *et al.* 2004, Brown-Douglas *et al* 2005, Dhakal *et al.* 2012].

Thus, the aim of the present study, involving large number of individuals, was to monitor the profile of oestradiol, testosterone and androstenedione every third month of life, from 6 to 18 months of age in growing foals, to create a reference level for maturing male and female Konik horses. The potential effects of the birth place (stable *vs*. forest), age and season were studied.

Material and methods

All procedures were accepted by the 3rd Local Commission for Ethics in Animal Experimentation, Warsaw, Poland. No other than routine veterinary procedures (blood sampling) were applied.

Animals

Fifty two Konik Polski horses, born in 2010 and 2011 at the Research Station of Polish Academy of Sciences, located in North-East part of Poland, were used (Tab. 1). For more than 10 generations, the breeding stock was maintained concurrently in two keeping systems – in a forest reserve as semi-feral familial groups and in traditional stables, with daily access to paddocks. Seasonally, stabled horses were pastured in close proximity to the forest reserve. The forest foals were born in five herds, consisting of one stallion and one to seven adult mares. All herds were dispersed on 1600 ha of coniferous and partly deciduous forest-located on a peninsula surrounded by three lakes. The foalings, both in the stables and the forest, were spread between April and June 2010 and 2011. The surplus offspring from the reserve were annually removed and after weaning reared together with stable-born foals.

Table 1. Distribution of 52 Konik foals according to sex and birth place

Birth place	Stable (N=27)		Forest (N=26)	
Sex	colts	fillies	colts	fillies
N	11	16	14	12

On the weaning day, the forest herds were closed in special enclosures and the weanlings were separated from adults in the weaning pen situated inside enclosures. Then, they were transported to the stable. Three weeks after weaning, forest foals were divided into sex groups and placed in new pens. The same day, the stable-born foals were weaned and joined their forest mates in four pens in the stable (two pens for colts and two pens for fillies). At the weaning time, the foals were from 31 to 40 weeks old. Until 18 months of age, all foals were stabled, turned out and pastured together in the sex groups.

Sampling procedure

The blood was drawn by jugular venipuncture from the stable-born foals at the age of about 6 months (n=27) and at the age of 9 months (one month after weaning) of the forest-born foals (n=26). The sampling was then repeated every three months *i.e*. at 9 (in stable-born foals), 12, 15 and 18 months of age. At the age of 18 months, one of the colts was dead. Moreover, due to technical reasons, not all samples could be used in the analysis, thus the exact number of individuals in each analysis is given in the result section.

Radioimmunoassay of plasma steroid hormones

Blood plasma concentrations of testosterone (T) androstenedione (A4) and oestradiol (E2) were measured as described by Dziadkowiec *et al*. [1982], Kotwica and Williams [1982] and Dusza *et al*. [1996]. Prior to the assays plasma samples were extracted with diethyl ether, mean extraction efficiency was 82.5%, 82.82% and 91.6% for testosterone, androstenedione and oestradiol, respectively.

Anti-T, anti-A4 and anti-E2 antibodies were characterized by Szafrańska *et al.* [2002]. The sensitivities of the assays for plasma testosterone, androstenedione and oestradiol were 1 pg/200µl, 2 pg/200µl and 2 pg/200µl of sample, respectively. Intra-assay coefficients of variation for T, A4 and E2 were 3.6%; 1.5% and 3.0%, respectively. Inter-assay coefficients of variation for T, A4 and E2 were less than 8%.

Statistical assay

As the variables mostly did not follow the normal distributions, the non-parametrical Mann-Whitney-Wilcoxon test (SAS System 9.3) was used to examine the differences in steroids' levels between sexes and stable *vs.* forest born foals at each age. Since the effect of the season was nested within the month of the year when samples were taken, these two factors were combined (age / month of sampling effect) and assessed applying Sign test (SAS System 9.3). Due to technical reasons, the effect of age / month of sampling on androstenedione level could not be calculated. Data are presented as means ± standard deviation and ranges.

Results and discussion

Steroid profile in colts and fillies

The profiles of sex steroids in young males and females are presented in Figure 1 whereas the means, standard deviations and ranges are shown in Table 2. The level of oestradiol differed between sexes only at the age of 12 months, whilst androgens were higher in males at every age group.

Forest *vs.* stable born foals

At all ages, the levels of oestradiol, testosterone and androstenedione did not differ between forest and stable-born colts and fillies, except for fillies at 18 months of age. Stable born 18 months old females had (or *tended* to have) higher plasma concentration of studied hormones (oestradiol: 21.01±2.99 and 23.82±3.13 pg/ml, z=1.97, P=0.0588; testosterone: 21.12 ± 5.78 and 28.08 ± 3.58 pg/ml, z=2.85, P=0.0082; androstenedione: 90.10±31.71 and 116.06±36.55 pg/ml, z=2.01, P=0.0535; forest and stable born fillies, respectively).

Age / season (month of sampling)

Being restricted to the sampling schedule (each 3rd month from 6 months of age), the effect of the season was evidently related to the age of growing foals. The highest levels of oestradiol in both colts and fillies were found at the age of 15 months, *i.e*. in the end of August and the beginning of September. In colts, oestradiol concentration differed significantly from its levels at 9, 12 and 18 months of age whereas in fillies it was different from all age groups except for 18 months (Tab. 2).

In colts, testosterone level was at its nadir at 6 and 9 months of age, then it rose abruptly at 12 months of age (May, Fig. 1B) and remained significantly elevated until

Table 2. The effects of age / month of sampling on sex steroids levels (pg/ml)

Age / month of sampling	Colts (N=25)				Fillies (N=28)			
	N	mean	SD	range	N	mean	SD	range
				Oestradiol				
6 / November	11	19.83	7.93	7.50-32.58	16	16.86^{aA}	6.40	9.53-30.34
9 / February-March	25	18.92^{A}	5.26	10.53-30.69	28	16.94^{A}	4.28	8.6-28.48
12 / May	25	18.62^{aA}	6.32	10.34-28.08	28	23.14^{BC}	5.83	10.82-33-92
15 / August-September	25	$\mathbf{25.18^{B}}$	**5.75**	9.33-39.11	28	$\mathbf{25.93^{B}}$	**4.69**	18.43-37.35
18 / October-November	24	21.68^{Ab}	4.44	4.87-32.33	28	21.91^{bC}	2.74	17.16-26.99
				Testosterone				
6 / November	11	31.28^{A}	13.74	11.88-58.81	16	15.28^{aA}	3.94	9.44-25.51
9 / February-March	25	45.16^{B}	18.90	21.88-95.95	27	18.20^{AB}	7.26	4.19-40.14
12 / May	25	$\mathbf{120.26^{C}}$	**84.78**	18.77-372.36	28	20.81^{b}	6.25	9.14-37.10
15 / August-September	25	117.03^{C}	86.64	24.16-316.55	28	$\mathbf{24.74^{B}}$	**5.30**	15.83-34.98
18 / October-November	24	95.21^{C}	71.52	11.55-216.86	28	23.86^{aB}	5.73	11.65-34.90
				Androstenedione				
6 / November	5	101.58	24.62	76.12-131.23	9	70.09	15.34	46.61-98.27
9 / February-March	12	105.07	29.72	71.88-144.69	15	67.37	17.68	26.87-85.36
12 / May	12	**192.75**	**80.93**	107.62-364.45	15	90.15	42.90	52.68-221.44
15 / August-September	12	**192.11**	**91.82**	78.11-436.41	28	**109.67**	**49.54**	57.40-234.50
18 / October-November	24	172.52	97.24	64.76-463.86	28	101.30	36.41	60.13-182.33

$^{aA...}$ In columns within each hormone means bearing different superscripts differ significantly at: small letters – $P<0.05$; capitals –$P<0.01$.
Values in bolds are the highest within each hormone level.

18 months (Tab. 2). In fillies, the highest concentration of this androgen was found in August-September, when the females were 15 months old. Its level remained almost unchanged until 18 months of age (Fig. 1B). Very similar phenomenon was observed for androstenedione (the highest levels at 12 and 15 months of age in colts and at 15 months in fillies, Fig. 1C).

The profiles of concentrations of plasma oestradiol, testosterone and androstenedione from 6 to 18 months of age in growing foals, established in the present study, could be used as the reference levels for maturing male and female Konik horses.

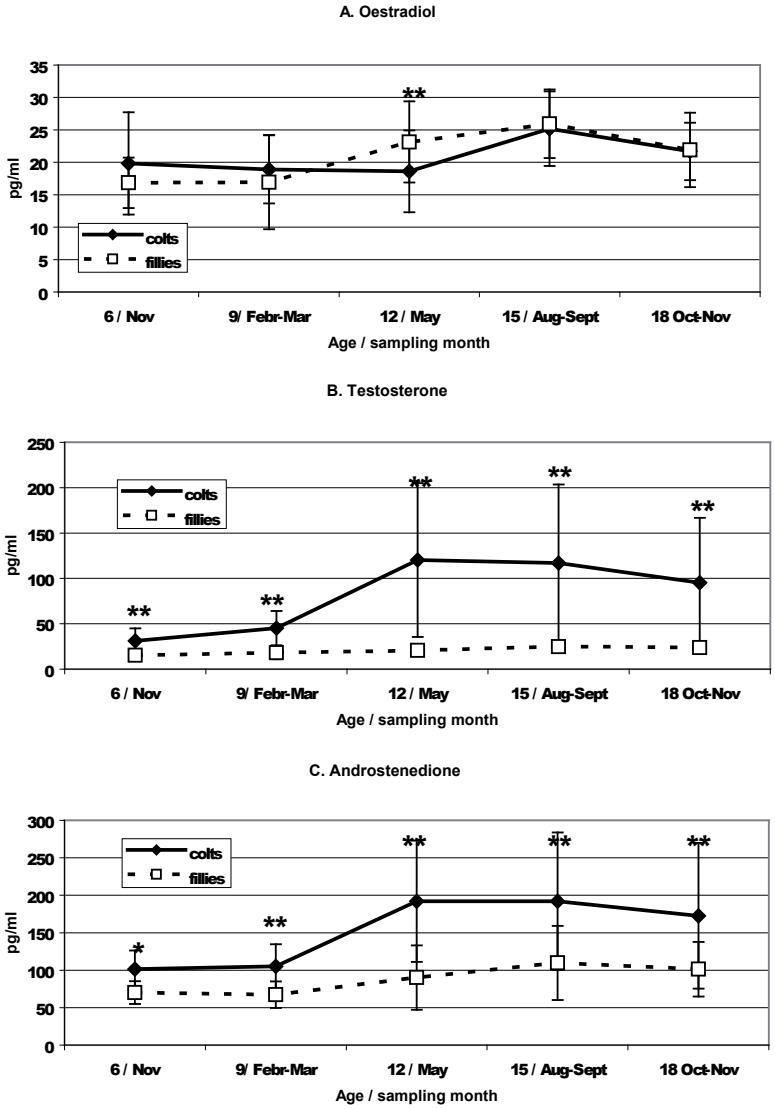

Fig. 1. Profiles of oestradiol (A), testosterone (B) and androstenedione (C) in 52 Konik horses colts and fillies at 6, 9, 12, 15 and 18 month of age.
*, **Denote significant differences between sexes at each age class at $P<0.05$ and $P<0.001$ respectively.

The levels and the profiles of oestradiol in fillies are in line with existing studies, mostly done on Thoroughbred foals [Lemazurier *et al*. 2002, Dhakal *et al*. 2012]. Unfortunately, our results can not be compared to those of Mongolian fillies as the results in Haffner's *et al*. [2010] study were below sensitivity of the assay. The

sampling schedule used in the present study did not allow precise monitoring of oestradiol variation in preovulatory period, so detailed activity of the ovaries could not be detected. Nevertheless, in some fillies of 15 months of age, the maximum range of oestradiol (37.35 pg/ml) closely approached the preovulatory level in adult mares (40 pg/ml; Medan *et al*. 2004). Successive increase of plasma concentration of oestradiol in maturing fillies could be observed from 9 to 15 months of age and at 12 months of age, it preceded similar increase in colts by three months. Similarly, androgens' levels peaked in colts at the age of 12 months, which confirms earlier studies on Thoroughbred colts [Lemazurier *et al*. 2002, Brown-Douglas *et al*. 2004, 2005]. According to previous foundings [Lemazurier *et al*. 2002, Dhakal *et al.* 2012], both colts and fillies were characterised with comparable oestradiol concentrations. In contrast, androgens concentration in fillies, although slightly increased in late summer and in autumn, did not show a spectacular variation, being lower than in colts from 6 months onwards, which agrees with the results of Lemazurier *et al*. [2002].

It is interesting in the present study that in peri-pubertal Konik horses concentrations of sex steroids were still high in late summer. This could have been detected due to the availability of animals up to 18 months of age. At this age, the average level of oestradiol in fillies was approaching the typical concentration reported for adult dioestrus mares [20 pg/ml; Medan *et al*. 2004]. Although the precise confirmation of puberty in fillies by determination of progesterone levels was not possible in our study, it can be supposed that at the age of 15-18 months young Konik fillies were already sexually mature. However, a very young free-roaming filly is rarely bred at this time. Usually, at the average age of 15.2 months (and first oestruses), a Konik filly is expelled by her sire from the natal band [Jaworski 2003]. The average age at first foaling in mares left in the forest as a breeding stock is 35.6 ± 12.1 months [Jaworski 2003] which indicates that first fertile breeding occurs when the fillies are on average 24-25 months old. This confirms social and behavioural regulation of reproduction in free-ranging horses [McDonnell 2000].

The present study did not generally confirm the differences in hormone levels between stable- and forest-born foals. The only difference concerned 18 months old fillies. No significant differences in physical development (data not shown) between stable and forest fillies were found. It can be hypothesised, that at sampling date more stable-born fillies were in preovulatory period, reflecting in higher androstenedione and testosterone concentrations, precursory hormones for oestradiol. The levels of steroids studied were comparable to the concentrations reported for Thoroughbred foals, a breed with a closed studbook and higher genetic distance to Koniks than between forest and stable born foals. Thus, the hypothesised effect of the genetic influence on the reproductive hormones could not be confirmed by the present study. It can be supposed that the differences in faecal androgens levels in stallions found previously by Opałka *et al.* [2010] were not due to genetic differences but most probably were related to behaviourally and socially different reproductive regimes between stable and forest horses (natural *vs*. human-controled). Similarly to other

reports on free-ranging horses, very young Konik stallions usually are not allowed to breed the mares, neither in their natal herd nor in other bands [Jaworski 2003]. According to the latter author, bachelors are expelled by fathers at 20.3 months of age (on average). First copulations by Konik forest stallions were observed as they were on average 48.3 months old [Jaworski 2003]. The formation of an own harem takes the stallion at least three years, and the success in band formation correlates with testosterone concentration [Khalil *et al.* 1998]. In Popielno reserve, the mean age of the stallion on herd formation was 67.7 months (48 – 108 months) [Jaworski 2003]. It was reported that in bachelor bands or in large stallions groupings (e.g. in stallions depôts) testosterone level fluctuates according to male social rank, being the highest in most dominating males [Kirckpatrick *et al*. 1976, McDonnell 2000]. Nevertheless, even if the stallion has the opportunity to breed, the level of testosterone in faeces is lower in younger as compared with older stable Konik stallions [Jezierski *et al*. in press]. Thus, it is not determined if high testosterone level predestines stallions to more masculine or aggressive behaviour enabling harem formation, or it is rather the opportunity of breeding that enhances testosterone surge [Khalil *et al*. 1998]. Further research is needed to elucidate mutual relations between testosterone levels and reproductive and social behaviour of stallions. The possibility of monitoring hormonal changes in feaces appears to be a promising non-invasive method in free-ranging horses [Schwarzenberger *et al*. 1996, Janowski *et al*. 2008, Opałka *et al*. 2010].

It can be concluded that the profiles of oestradiol, testosterone and androstenedione in a large cohort of maturing Konik Polski males and females are now available as breed reference levels. The levels of studied hormones are comparable to these reported earlier in Thoroughbred foals. This is, to our knowledge, the first report on steroid profiles in other than Thoroughbred breed, involving large number of horses followed up to 18 months of age.

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