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Various factors affecting the alpha1-antitrypsin level in Thoroughbred foals

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Abstract: *Various factors affecting the alpha*1*-antitrypsin level in Thoroughbred foals*. Acute phase proteins (APP) are an integral part of the acute phase response. Alpha1-antitrypsin (AAT) is considered to be one of the most important acute-phase protein activated by trauma, stress, or inflammatory processes. The objective of the present study was to estimate the impact of various factors (sex, month of life and sire effect) on concentration of alpha1-antitrypsin in serum of Thoroughbred foals. A total of 624 samples, collected from 39 foals were obtained in monthly intervals from first to 16th month of life and measured by STIC method (specific trypsin inhibitory captivity). The obtained results indicated the significant impact of analyzed age periods on the AAT level. Furthermore, the variation in AAT level in analyzed periods corresponded to significant changes in foals diet and maintaining. Alpha1-antitrypsin concentration was also affected by sire effects and sex of foals. In the most investigated age periods, the impact of sire on alpha1-antitrypsin content in serum of his progeny has been shown. The obtained results might be useful in explanation of differences in serum AAT concentration in foals during early ontogenesis which probably is a critical period that has an influence on racing performance of young horses.

Key words: alpha1-antitrypsin, acute phase proteins, Thoroughbred

INTRODUCTION

The Thoroughbred (TB horses) is one of the most valuable horse breed in the world. As a registered race horse, the stud books trace back up to 18th century (An Introduction... 1791). Since that, TB horses were subjected to artificial selection pressure and planned breeding for superior athletic and racing performance. Furthermore, horse in general has been used as a large animal model for investigation of biochemical, physiological and genetic adaptation to stress factors (McGivney et al. 2009, Fureix et al. 2012). During the growth period important changes in young developing organisms occurred and many studies have been performed to known physiological mechanisms of these modifications (Paltrinieri et al. 2008, Lepeule et al. 2009, Duesterdieck-Zellmer et al. 2014). One of the most important blood proteins are acute phase proteins (APP) which are an

integral part of the acute phase response. Information about changes in the serum APP proteins concentration are used in diagnosis, prognosis and assessment of animal health (Cray et al. 2009). Age related changes in reference values of these biochemical parameters have been described in several equine breeds (Brommer et al. 2001, Čebulj-Kadunc et al. 2002, Muñoz et al. 2012).

The alpha1-antitrypsin enzyme (AAT), known also as alpha1-proteinase inhibitor (A1PI, APi), is a plasma glycoprotein that belongs to SERPIN superfamily. Like in humans, equine AAT is synthesized in liver, in the Islets of Langerhans and it is believed that other immunolocalisation of this protein is similar to humans' (Dagleish et al. 1998). The target proteinase is neutrophil elastase (NE) which is released during the inflammatory response and uncontrolled causes degradation of extracellular matrix (Janof 1985). A genetic disorder of AAT deficiency (AATD) has been widely described in humans. Mutation in gene encoding alpha1-antitrypsin, including single point mutations, insertions and deletions, lead to protein retention in the endoplasmic reticulum and failure of secretion consequently resulting in low level of ATT in plasma and lungs (Stockley and Turner 2014). The equine APi system has been shown to be controlled by four closely linked loci Spi1, Spi2, Spi3, Spi4 which probably derive from the same ancestral gene that encodes human AAT. Further studies described evidence that Spi1 proteins are the equivalent of human APi (Patterson et al. 1991). Isolation of three different functional inhibitors from horse plasma showed that two of them can inactivate horse NE (Potempa et al. 1991). Whereas human APi is a one oxidation sensitive protein, horse APi includes five isoforms and only one of them is oxidation sensitive (Patterson and Bell 1989, Patterson et al. 1991). Study on horse neutrophils provide evidence that synthesis and release of APi also occur in mature equine neutrophils and concurrent extracellular release of neutrophil elastase. The reactive oxidative intermediates from stimulated equine neutrophils would not inactivate all APi, so level of neutrophil elastase inhibition maintain protection from proteolytic damage. This could be an explanation of differences between human and horse neutrophil activity in pulmonary pathology (Dagleish et al. 2003). For the time, the possible hereditary nature of recurrent airway obstruction (RAO) has been indicated due to the stallion which half of descendants have been affected (Schäper 1939). Further studies revealed that risk of RAO appearance is 3.2 higher (p >0.005) when one parent is affected and is 4.6 higher when considering both parents (Marti et al. 1991). To date, there are very limited information about biological significance and deficiency of equine alpha1-antitrypsin during growth and development of foals.

The objective of the present study was to estimate the impact of various factors (sex, month of life and sire effect) on concentration of alpha1-antitrypsin in

the serum of Thoroughbred foals. The obtained results might be useful in explanation of differences in serum AAT concentration in foals during early ontogenesis which probably is a critical period that influence racing performance of young horses.

MATERIAL AND METHODS

Animals and samples collection

Blood samples were obtained from 39 Thoroughbred foals born in the same year, raised in two stud farms with similar agronomic conditions. Study sample of 20 fillies and 19 colts were divided into four groups according to their pedigrees (foals do not have a common mothers). Each group represented foals by one father and the foals sex ratio was 1 : 1. Samples were collected in monthly intervals from the first to 16th month of life (a total of 624 samples were collected). Foals were dewormed and vaccinated at the proper time. They were fed with the diet recommended for foals, accurate to the state of development and season.

Serum alpha1-antitrypsin measurements

Blood samples were collected in to sterile tubes without anticoagulant by jugular venipuncture, then samples were centrifuged at 4,380 g for 5 min, and the serum was used for measurements of ATT.

Concentration of trypsin inhibitory capacity (TIC) serum was measured according to principle of the method by using *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA, Sigma) as substrate primarly described by Dietz and coworkers (Dietz et al. 1976). Reduction in tryptic activity after the addition of plasma to a standard trypsin solution was measured. The standard curve was obtained by the use of scalar concentration of trypsin (bovine pancreas trypsin, Sigma 9300 Unit BAEE). For the determination of TIC (0.02ml), buffer (0.02M $CaCl_2$– 0.1 M Tris, pH 8.2; 3.7 ml) and trypsin (10.0 mg in 50.0 ml 0.0025N HCl; 0.2 ml, about 40 μg of trypsin), were pre-incubated in a water bath. After adding the substrate (4.0 ml) and 10 min of incubation, the reaction was stopped by the addition of 30% acetic acid (1.0 ml). The result of the reaction was read at a photometer (410 nm wavelength). The TIC parameters were calculated according to the reduction of enzyme activity by the serum (1 mg of trypsin per 100 ml of serum). The determined values of AAT level and TIC were used for the calculation of the specific AAT activity (1 mg of trypsin inhibited by 1 mg of AAT).

Statistical analysis

The data were analyzed with the use of GLM procedure (SAS Institute, Cary, NC, USA; ver 9.2). Two different linear models were applied. Model 1 was used for general analysis whereas effect of sex on AAT was tested by means of Model 2:

Model 1:

$$y_{ijk} = o_i + c_j + (o \cdot c)_{ij} + z_{ik} + \varepsilon_{ijk}$$

Model 2:

$$y_{ijk} = p_i + c_j + (p \cdot c)_{ij} + z_{ik} + \varepsilon_{ijk}$$

where:

o – fixed effect of the stallion;

c – fixed effect of the measurement time on j time;

$(o \cdot c)_{ij}$ – interaction between sire and time effect;

z_{ik} – fixed foal effect;

p – fixed sex effect;

ε – random error.

The averages for individual measurement time points were compared using profile, mean and helmert contrasts. Profile contrasts were used to compare adjacent measuring time points, while each data time point with the average of all points were compared using the mean contrasts. Finally, Helmert contrasts were applied to compare each time point with the average points following it. To confirm the significance of impact of the factors on AAT levels in the analyzed periods three orthogonal contrasts have been analyzed:

- Contrast A was sire IV vs all other sires;
- Contrast B was sire III vs sires I and II;
- Contrast C was sire II vs sires I and I.

Differences between sires of every individual measuring time point were tested using orthogonal contrasts, including sire by analyzed periods interaction effect.

RESULTS AND DISCUSSION

Alpha1-antitrypsin is considered to be one of the most important acute-phase protein activated by trauma, stress, or inflammatory processes. Due to inhibition of a wide variety of proteases, alpha1--antitrypsin is also called alpha1-proteinase inhibitor (A1PI; APi). This inhibitor is a critical element which protects lung tissue from uncontrolled, destructive influence of proteolytic enzymes of inflammatory cells, especially neutrophil elastase. The low concentration of AAT protein in the respiratory system can lead to the gradual and irreversible reduction of lungs elasticity. The excessive neutrophil elastase activity results in degradation of elastin, the main component of elastic fiber, and other extracellular matrix components in the lower respiratory tract (Stockley 2000). These changes would be related to respiratory complications characterized by inflammation or chronic obstructive lung disease.

As previously described equine APi are composed of four or five plasma glycoproteins named serine proteinase inhibitor (Spi) 1, 2, 3A, 3B, 4. The Spi1 proteins are the equivalent of human APi (Patterson et al. 1991, Potempa et al. 1991) and Spi1, Spi3A, Spi3B, Spi4 have the ability to inhibit trypsin (Patterson et al. 1991). Whereas human APi is a one oxidation sensitive protein, horse APi includes five isoforms and only one of them is oxidation sensitive (Patterson and Bell 1989, Patterson et al. 1991). Study on horse neutrophils pro-

vide evidence that synthesis and release of APi also occurs in mature equine neutrophils and concurrent extracellular release of neutrophil elastase. The reactive oxidative intermediates from stimulated equine neutrophils would not inactivate all APi, so level of neutrophil elastase inhibition maintain protection from proteolytic damage. This could be an explanation of differences between human and horse neutrophil activity in pulmonary pathology (Dagleish et al. 2003).

In the present study, the statistical analysis showed that all tested effects (sex, month of life and stallion effect) highly significantly affected AAT levels. Mean levels of AAT in serum of fillies and colts in the first 16 months of life were estimated. Concentration of antitrypsin ranges between 1.3 and 1.9 mg/ml (Fig. 1). The obtained levels of AAT were lower than previously estimated by different authors: 1 and 2.1–4.0 mg/ml (Patterson et al. 1991, Pellegrini 1994, Dagleish et al. 2000). On the other hand, the presented discrepancies in antitrypsin concentration may result from different methods used to estimation of AAT levels (enzymatic assay and immunoassay). Furthermore, immunoassay method which detects protein level based on the binding with specific antibody, could be influenced by different factors.

In our results, the highest fluctuation in serum AAT concentrations in foals has been observed in the first 6 months of life. Minimum antitrypsin level has been detected in the fifth and a maximum in the third month of life. The observed decrease of AAT concentration is probably a consequence of managing procedures when foals are changing rearing at the stable environment on grazing. Subsequent decrease of AAT content in serum was noted at around eighth month of life which was presumably associated with weaning procedures. Weaning occurs in foals at the eighth month and is the most stressful period (Apter and Houshholder 1996). It is associated with the loss

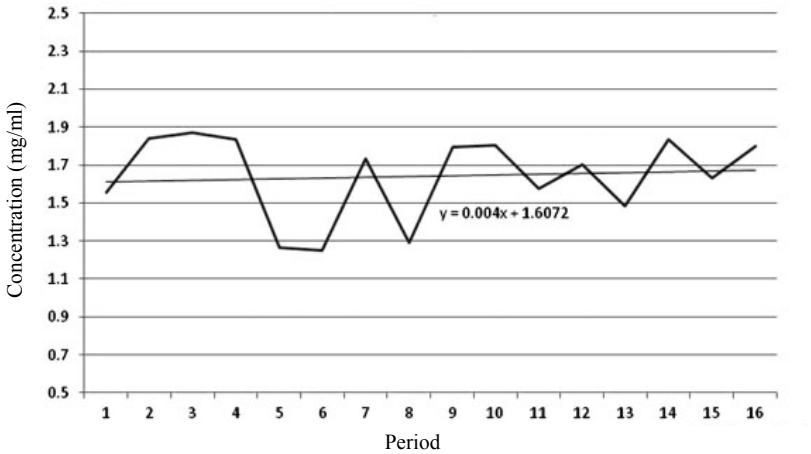

FIGURE 1. Content in serum of alpha1-antitrypsin in foals from first to 16th month

of weight (Waran et al. 2008), increase of immune response and increase of cortisol level (Malinowski et al. 1990). The presented variations in antitrypsin levels in analyzed periods were also confirmed by significance of orthogonal contrasts (p <0.01) (Table 1). In horses, the different acute-phase proteins (APP) manifest a various pathological processes, such as inflammatory process, stress, infections caused by bacteria, viruses, parasites or trauma surgery. In foals, Satué et al. (2013) showed significant impact of the age on another equine APP protein (amyloid type A; SAA), while gender probably does not affect basal levels of this protein. Furthermore, several previous studies confirmed significantly higher level of amyloid A in horses older than 21 months compared to foals at 18 or 12 months (Nunokawa et al. 1993, Satoh et al. 1995). It is proven that SAA concentration was very high in horses with clinical signs of inflammation and also increased after surgery treatment (Eckersall 1995).

TABLE 1. Differences between mean of ATT levels (mg/ml) of foals sired by different stallions in individual measurement points

Point[a]	A[b]		B[c]		C[d]	
	F	*p*	*F*	*p*	*F*	*p*
1	9.510	**	78.880	**	0.350	–
2	16.340	**	6.010	*	0.160	–
3	30.560	**	5.360	*	0.010	–
4	1.390	–	0.380	–	0.360	–
5	2.430	–	19.100	**	1.530	–
6	8.150	**	32.180	**	0.660	–
7	0.410	–	7.290	**	0.430	–
8	51.460	**	23.760	**	0.320	–
9	28.400	**	0.820	–	0.250	–
10	23.300	**	22.150	**	1.410	–
11	8.220	**	0.000	–	1.010	–
12	12.050	**	7.400	**	0.700	–
13	21.340	**	0.830	–	0.250	–
14	9.130	**	0.270	–	0.000	–
15	15.440	**	0.070	–	0.710	–
16	34.230	**	7.180	**	1.150	–

[a]Measurement point which is the month of foals life; [b]A – sire IV vs I, II, III; [c]B – sire III vs I, II, [d]C – sire I vs II; *$p \leq 0.05$; **$p \leq 0.001$.

Basic statistical analysis showed that concentration of alpha1-antitrypsin level was also influenced by sire effects and sex of foals. In the most investigated age periods, the impact of sire on alpha1-antitrypsin content in serum of his progeny has been shown (Fig. 2). Highly significant differences were found between AAT levels in progeny of sire IV vs sires III, II, I, and sire III vs sires II, I (p >0.05). Whereas the AAT mean serum level of progeny of sire IV ranging between 0.7 and 1.7 mg/ml (mean 1.4), for progeny of sire III 0.9––2.1 mg/ml (1.7), for progeny of sire II 1.0–2.1 mg/ml (1.7), for progeny of sire I 0.98–2.20 mg/ml (1.8).

Furthermore, the differences in antitrypsin levels between foals of four stallions in individual months of life were observed. Interesting results were obtained for the offspring of stallion IV which showed highly significant differences in AAT level from the first to third month of life, six month of life and further, from eighth month until end of the experiment. On the other hand, foals of stallion III showed highly significant differences in AAT concentration at the first month of life, fourth to eight month, 10, 12 and 16 months (Table 2). The orthogonal contrasts also illustrated that not

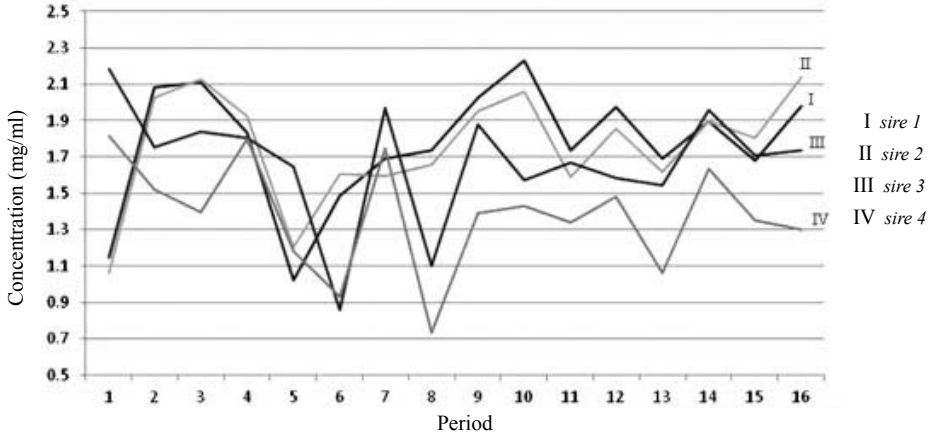

FIGURE 2. Content in serum of alpha1-antitrypsin in sires I–IV from first to 16th month

TABLE 2. Differencies in mean alpha1-antitrypsin levels in foals sired by different stallions

Contrast	*SS*	*MS*	*F*	*p*
A[a]	17.42	17.42	181.57	**
B[b]	0.81	0.81	8.41	**
C[c]	0.01	0.01	0.16	–

[a]A – sire IV vs I, II, III; [b]B – sire III vs I, II; [c]C – sire I vs II; $^{**}p \leq 0.001$.

every sire influences serum antitrypsin content in their progeny. The statistical significance was obtained only for stallions III and IV when compared to the mean AAT concentration of others tested stallions (Table 3). The association of only some stallions with alpha1--antitrypsin levels in offspring indicated the presence of some inheritance factors which may determinate the above feature. It seems important to performed future research on molecular basis of AAT content in horses, what may be useful in breeding selection. In humans, to diagnose the reason of AAT deficiency, few polymorphic sites have been detected and results confirmed the significant association of alpha1-antitrypsin phenotypic isoforms with genetic variation (Wu and Foreman 1991, Bornhorst et al. 2007). To date, there is no data which reported influence of sex on APi concentration in blood serum in horses. In dogs, Hughes et al. (1995) showed that AAT concentration was significantly higher in healthy, sexually inaction females when compared to spread females, sexually inaction males or castrated males. The authors suggested that antitrypsin level may be affected by estrogen hormones. On the other hand, in studies conducted on humans, Bornhorst et al. (2013) indicated that age, race, and sex had only slight effects on the median 95% serum antitrypsin concentration. Furthermore, acute phase proteins are produce due to the stress response. The higher level of AAT in colts is related with stress induced testosterone stimulation on adrenaline. In our study, the GLM procedure confirmed significant effect of gender

TABLE 3. Differences in antitrypsin levels depends on sex in measurement points

Point[a]	Fillies		Colts		Contrast	
	LSM	*SE*	*LSM*	*SE*	*SS*	*p*
1	1.504	0.085	1.588	0.079	0.072	–
2	1.658	0.085	1.981	0.079	1.067	**
3	1.838	0.085	1.886	0.079	0.024	–
4	1.749	0.085	1.892	0.079	0.207	–
5	1.229	0.085	1.284	0.079	0.030	–
6	1.161	0.085	1.311	0.079	0.229	–
7	1.727	0.085	1.723	0.079	0.000	–
8	1.176	0.085	1.372	0.079	0.394	–
9	1.785	0.085	1.792	0.079	0.001	–
10	1.673	0.085	1.905	0.079	0.550	*
11	1.476	0.085	1.650	0.079	0.311	–
12	1.493	0.085	1.865	0.079	1.417	**
13	1.252	0.085	1.670	0.079	1.777	**
14	1.762	0.085	1.882	0.079	0.146	–
15	1.504	0.085	1.725	0.079	0.499	–
16	1.568	0.088	1.975	0.079	1.640	**

[a] Measurement point which is the month of foals life; *$p \leq 0.05$; **$p \leq 0.001$.

on AAT content in foal serum ($p \leq 0.05$) (Fig. 3). Generally, in the most of investigated periods fillies were characterized by lower value of AAT concentration comparing to colts. The increase of differences in antitrypsin levels dependent on sex was observed from the 9th to 10th month of life and the biggest discrepancies between fillies and colts were obtained at 12th, 13th, and 16th month of life (Fig. 3, Table 4). At 12th–13th month, the Thoroughbred horses enter puberty and

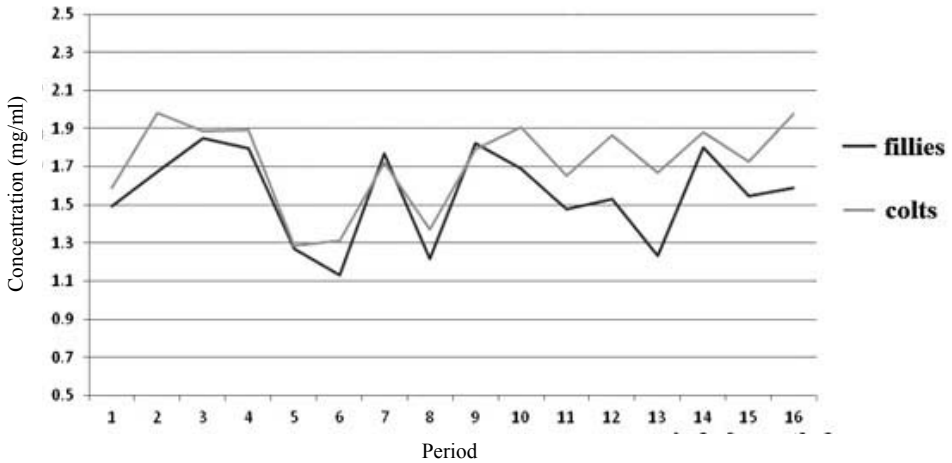

FIGURE 3. Content in serum of alpha1-antitrypsin in fillies and colts from first to 16th month

TABLE 4. Variation in mean alpha1-antitrypsin levels (mg/ml) in analyzed periods

Point[a]	*LSM*	Type of orthogonal contrast[b]					
		Profile[c]		Mean[d]		Helmert[a]	
		SS	*p*	*SS*	*p*	*SS*	*p*
1	1.549	1.695	**	0.354	–	0.354	–
2	1.844	0.018	–	1.708	**	1.613	**
3	1.874	0.053	–	2.251	**	2.429	**
4	1.822	6.397	**	1.356	**	1.809	**
5	1.249	0.002	–	6.399	**	5.169	**
6	1.239	4.761	**	6.737	**	6.540	**
7	1.733	3.622	**	0.349	–	0.181	–
8	1.302	4.930	**	4.789	**	5.661	**
9	1.805	0.004	–	1.112	**	0.434	*
10	1.820	1.064	**	1.323	**	0.740	**
11	1.586	0.293	–	0.127	–	0.335	–
12	1.709	0.924	**	0.189	–	0.021	–
13	1.491	2.274	**	0.941	**	1.904	**
14	1.832	0.863	**	1.519	**	0.432	*
15	1.622	0.510	*	0.015	–	0.510	*
16	1.785	–	–	0.836	**	–	–

[a]Measurement point which is the month of life foals; [b]The averages for individual measurement time points were compared using profile, mean and helmert contrasts; [c] Profile contrasts were used to compare adjacent measuring time points; [d]Mean contrasts were used to compare each data time point with the average of all points; [e]Helmert contrasts were applied to compare each time point with the average points following it.
*$p \leq 0.05$; **$p \leq 0.001$, $SE = 0.05$.

the separation of colts from fillies was performed, which may also explain the greatest variation of serum AAT content between genders at these periods. The obtained results might indicate that hormones involved with sexuality, especially estrogens, play important role in antitrypsin regulation.

CONCLUSIONS

According to very limited information about physiological basis of deficiency of equine alpha1-antitrypsin, the presented results would be helpful in understanding equine AAT regulation. The present study confirmed the significant impact of sex, age and sire effect on concentration of alpha1-antitrypsin in serum in Thoroughbred foals. The variation in AAT level in analyzed periods corresponded to significant changes in foals diet and maintaining. Different effect of investigated stallions on alpha1-antitrypsin level in their offspring indicated the need of future research concerning genetic determinants of AAT concentration.

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Streszczenie: *Wpływ różnych czynników na poziom alfa*1*-antytrypsyny u źrebiąt pełnej krwi angielskiej*. Białka ostrej fazy (APP) są integralną częścią tzw. odpowiedzi ostrej fazy na stan zapalny. Alfa1-antytrypsyna (ATT) jest uważana za jedno z najważniejszych białek ostrej fazy aktywowane przez uraz, stres lub procesy zapalne. W związku z tym celem niniejszego badania była ocena wpływu różnych czynników (płeć, miesiąc życia i ojciec) na stężenia ATT w surowicy krwi źrebiąt pełnej krwi angielskiej. Materiał do badań stanowiły 624 próbki krwi, zebranych od 39 źrebiąt będących potomstwem czterech ogierów, uzyskanych w odstępach miesięcznych od pierwszego do 16. miesiąca życia. Stężenie ATT oznaczono metodą STIC. Uzyskane wyniki wskazały na znaczący wpływ wieku źrebiąt na poziom AAT. Co więcej różnice w poziomie AAT w analizowanych okresach przypadały w okresach znaczących zmian u źrebiąt. Ponadto w większości badanych okresów wykazano wpływ ojca na stężenie ATT u potomstwa. Uzyskane wyniki mogą być przydatne w celu wyjaśnienia różnic w koncentracji AAT w surowicy krwi u źrebiąt w okresie wczesnej ontogenezy, która jest krytycznym okresem mającym wpływ na wyniki użytkowe młodych koni.

Słowa kluczowe: alfa1-antytrypsyna, białka ostrej fazy, pełna krew angielska

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