



## Correlation of maternal concentrations of plasma testosterone with fetal sex in horses

Eduarda Maciel Busato<sup>1\*</sup>  Romildo Romualdo Weiss<sup>1</sup>   
Ana Claudia Machinski Rangel de Abreu<sup>1</sup>  Tácia Gomes Bergstein-Galan<sup>1</sup>   
Felipe Augusto Beckert Marcondes<sup>2</sup>  Luiz Ernandes Kozicki<sup>3</sup>   
Vanete Thomaz Soccol<sup>1</sup>  Peterson Triches Dornbusch<sup>4</sup> 

<sup>1</sup>Programa de Pós-graduação em Engenharia de Bioprocessos e Biotecnologia, Universidade Federal do Paraná (UFPR), 81530-980, Curitiba, PR, Brasil. E-mail: eduarda.busato@gmail.com. \*Corresponding author.

<sup>2</sup>Faculdade Evangélica do Paraná (FEPAR), Curitiba, PR, Brasil.

<sup>3</sup>Pontifícia Universidade Católica do Paraná (PUCPR), Curitiba, PR, Brasil.

<sup>4</sup>Departamento de Medicina Veterinária, Universidade Federal do Paraná (UFPR), Curitiba, PR, Brasil.

**ABSTRACT:** *The objectives of this study were to evaluate the correlation of fetal sex and plasma testosterone concentrations between the 5<sup>th</sup> and 8<sup>th</sup> months of pregnancy in mares and to verify the applicability of this test to predict fetal sex. Blood samples were collected from 21 mares at 30-day intervals of between 150 and 240 days of pregnancy. Plasma testosterone was determined by radioimmunoassay and the sex of the foals confirmed at birth. The levels of maternal testosterone were higher in mares carrying female fetuses at months 5 and 8 ( $P < 0.05$ ). Limit values were determined by analyzing the receiver operating characteristic (ROC) estimates: 35.5 pg/mL and 40 pg/mL for the 5<sup>th</sup> and 8<sup>th</sup> month, respectively. For the mares with plasma testosterone values equal to or above the threshold, gestation of female foals was predicted, and for those with plasma testosterone below the threshold values pregnancy of male foals was predicted. In the 5<sup>th</sup> month, the predictive values for male and female fetuses were 70% and 88.9%, respectively; the detection rates were 87.5% and 72.7%, and the total accuracy of the examination was 78.9%. In the 8<sup>th</sup> month, the predictive values for male and female fetuses were 80% and 90%, respectively; the detection rates were 88.9% and 81.8%, and the total accuracy of the examination was 85%. It was concluded that there was a correlation between fetal sex and plasma testosterone concentrations in pregnant mares. Prediction of fetal sex based on plasma concentrations of maternal testosterone can be performed in months 5 and 8 with 78.9% and 85% accuracy, respectively.*

**Key words:** fetal sexing, horses, equine reproduction.

## Correlação das concentrações plasmáticas de testosterona materna com o sexo fetal em equinos

**RESUMO:** *Os objetivos do estudo foram avaliar a correlação do sexo fetal com as concentrações plasmáticas de testosterona entre o 5<sup>o</sup> e o 8<sup>o</sup> mês de gestação na égua e verificar a aplicabilidade deste exame para a predição do sexo fetal. Amostras de sangue foram coletadas de 21 éguas, com intervalos de 30 dias, entre 150 e 240 dias de gestação. A testosterona plasmática foi determinada por radioimunoensaio e o sexo dos potros foi confirmado ao nascimento. Os valores de testosterona materna foram superiores nas éguas gestando fetos fêmeas aos cinco e oito meses ( $P < 0.05$ ). Através da análise da curva ROC (receiver operating characteristic) foram determinados valores limites de 35,5 pg/mL e 40 pg/mL para o 5<sup>o</sup> e o 8<sup>o</sup> mês, respectivamente. Éguas com testosterona plasmática igual ou acima dos valores limites foram preditas como gestando fêmeas e éguas com testosterona plasmática abaixo dos valores limites foram preditas como gestando machos. Aos cinco meses, os valores preditivos para fetos machos e fêmeas foram 70% e 88,9%, respectivamente; as taxas de detecção foram 87,5% e 72,7% e a acurácia total do exame foi de 78,9%. Aos oito meses, os valores preditivos para fetos machos e fêmeas foram 80% e 90%, respectivamente; as taxas de detecção foram 88,9% e 81,8% e a acurácia total do exame foi de 85%. Conclui-se que houve correlação entre o sexo fetal e as concentrações de testosterona plasmática em éguas prenhes. A predição do sexo fetal baseada nas concentrações plasmáticas de testosterona materna pode ser realizada aos cinco e oito meses de gestação com 78,9% e 85% de acurácia, respectivamente.*

**Palavras-chave:** sexagem fetal, cavalos, reprodução equina.

## INTRODUCTION

Prenatal diagnosis of fetal sex (fetal sexing) is routinely performed in human medicine; whereas, in veterinary medicine is primarily employed in cattle

and horses. Interest in fetal sexing varies according to the species and/or breed in question. In dairy cattle, the preference is for females, while in beef cattle, males are generally preferred due to their greater weight and better feed conversion (KIBUSHI et al., 2016). In

wild species kept in captivity, fetal sexing can be used as a tool for management, which is often different between males and females (DUER et al., 2002).

In horses, the interest for foals of a particular sex varies according to breed, activity and preference of the owner. In polo ponies, there is a preference for females (PASHEN et al., 1993; PANARACE et al., 2014), while in racehorses, males are usually preferred (CHEZUM & WIMMER, 1997). Although, fetal sexing is an examination to diagnose but not choose the fetal sex, it can influence strategic decisions made by the breeder. Mating planning, whether or not to put pregnant mares to auctions or an animal's market value or insurance value may be directly affected by the sex of the animal (MCGLADDERY, 2011; AURICH & SCHNEIDER, 2014).

In horses, fetal sexing is commonly performed by reproductive ultrasonography. There are two approaches: the first, based on the identification and position of the genital tubercle of the fetus, is performed by transrectal ultrasonography (TRUS), preferably between days 59 and 68 of pregnancy (CURRAN & GINTHER, 1989); the second is based on the evaluation of the fetal gonads and external genitalia and can be performed by TRUS (between days 90 and 150, approximately) or transabdominal ultrasonography (until day 220 of pregnancy, approximately) (RENAUDIN et al., 1997, 1999; BUCCA, 2005; LIVINI, 2010).

A molecular technique using the polymerase chain reaction (PCR) has been described to perform fetal sexing in the last 3 months of pregnancy in horses. This method has 95% accuracy and is based on the detection of circulating free fetal DNA in maternal blood. This examination is less invasive than the aforementioned ones because it only requires the collection of mare's blood, eliminating the risks inherent in palpation per rectum (DE LEON et al., 2012). However, the application of this fetal sexing method in the field is still limited and restricted to research work.

Despite the options available for the determination of fetal sex in pregnant mares, this test is still relatively underused in equine reproduction. Current techniques require ultrasound equipment and examiner experience, which limit their application. The use of ultrasonography could be more intensively employed if its accuracy could be increased, especially when performed as a single evaluation and under field conditions (MARI et al., 2002; BUCCA, 2005; AURICH & SCHNEIDER, 2014).

Alternatives to traditional methods of fetal sexing have been described in humans

(MEULENBERG & HOFMAN, 1991), elephants (DUER et al., 2002) and cows (KIBUSHI et al., 2016) and are based on maternal levels of blood testosterone. In a study conducted by MEULENBERG & HOFMAN (1991), women carrying male fetuses presented a gradual increase in testosterone levels during pregnancy, while those carrying female fetuses presented decreasing levels of testosterone after the first trimester.

In Asian elephants, serum levels of testosterone are significantly higher in animals carrying male fetuses than in those carrying female fetuses, from the second trimester of pregnancy (DUER et al., 2002). In cows, the sex of the fetus also influences the levels of plasma testosterone, which are higher in cows carrying male calves, from the second half of pregnancy (KIBUSHI et al., 2016).

According to DUER et al. (2002), testosterone production in placental mammals is associated with differentiation of the reproductive tract. The experimental findings by KIBUSHI et al. (2016) corroborated this statement, as these researchers believe that the highest testosterone concentrations in cows gestating male fetuses after mid-pregnancy are related to the passage of fetal androgens through the placenta. MEULENBERG & HOFMAN (1991) also believed that a proportion of maternal testosterone in pregnancy is of fetal origin. According to these researchers, differences related to fetal sex indicated that unbound testosterone crosses the placenta of the male fetus into the maternal circulation, while the opposite flow occurs in pregnancies of female fetuses.

An experiment conducted by SILBERZAHN et al. (1984) measured plasma testosterone concentrations during mares' pregnancy. According to the authors, mares have a peak in plasma testosterone levels in the seventh month of pregnancy. Although, results of the study demonstrated that at seven months of pregnancy mares gestating female fetuses had higher plasma testosterone concentrations than those gestating male fetuses, the difference was not significant. To date, no studies have been conducted in mares to validate the determination of maternal testosterone as a marker of fetal sex. The hypothesis of the present study is that plasma testosterone is higher in mares gestating male fetuses than in those gestating female fetuses, in agreement with the results reported in other mammals. Thus, the objectives of the experiment were to assess the correlation of fetal sex with plasma testosterone concentrations between the 5th and 8th months of pregnancy in the mare and to verify the applicability of this test for the prediction of fetal sex.

## MATERIALS AND METHODS

Twenty-one Crioulo mares, aged 4 to 17 years (mean  $8.8 \pm 4.2$  years) were used, from a farm located at latitude  $25^{\circ}32'84''\text{S}$  and longitude  $49^{\circ}87'84''\text{W}$ . Mares were mated or artificially inseminated using five different stallions of the same breed after follicular control, and ovulation was detected by TRUS and the date noted. Monthly blood collections were performed from the 5<sup>th</sup> month until the 8<sup>th</sup> month of pregnancy. The mares were restrained in a horse crush to perform the collections by jugular venipuncture, using a syringe (10 mL) and disposable needle (40 mm  $\times$  12 mm). Samples were deposited in blood collection tubes containing EDTA, homogenized and refrigerated at 5 °C during transport.

In the laboratory, the collected samples were kept at room temperature for 10 minutes and then centrifuged at 5000 rpm for separation of plasma. The plasma aliquots were placed in identified Eppendorf tubes, frozen and stored at -20 °C until determination of testosterone levels. Each sample was categorized according to the months of pregnancy, as follows: 5<sup>th</sup> month (days 144 to 154 – mean  $150 \pm 3.4$  days), 6<sup>th</sup> month (days 179 to 188 – mean  $182 \pm 2.7$  days), 7<sup>th</sup> month (days 209 to 218 – mean  $212 \pm 2.7$  days) and 8<sup>th</sup> month (days 237 to 255 – mean  $243 \pm 3.9$  days), considering day zero (D0) as the day of ovulation.

The samples collected at months 5, 6, 7 and 8 of pregnancy were used for testosterone radioimmunoassay (RIA). A commercial kit from Immunotech (Beckman Coulter, RIA Testosterone, direct – REF IM 1119) was used for the determination of plasma testosterone. The sensitivity of the assay is 20 pg/mL for testosterone. The laboratory procedures for the assay were performed according to the manufacturer's instructions. The intra-assay coefficient of variation was 12.8%.

Results of the measurements were categorized according to the pregnancy month and the foal's gender. The Grubb's test (Graphpad software) was applied to each category for detection and exclusion of outliers. Analysis of variance (ANOVA) was performed to verify the variation in maternal testosterone levels according to the fetal sex and month of pregnancy. The Tukey post-test was performed for repeated measurements in order to analyze the relationship of the pregnancy month to testosterone levels. The unpaired t-test was used to evaluate the influence of fetal sex on maternal serum testosterone. The P-values less than 0.05 were considered significant for all statistical analyses.

Threshold values were established in the months in which there was a statistical difference in testosterone concentrations according to the fetal sex. These values were determined using the receiver operating characteristic (ROC) curves produced by the statistical analysis software SigmaPlot TM 12.0 (Systat software, San Jose, CA). Predictive values for female and male fetuses were determined based on the threshold. These predictive values were calculated by dividing the number of mares carrying fetuses of a given sex by the number of mares predicted for the same sex by their testosterone levels (Figure 1a–b). The detection rate for male or female fetuses was defined as the number of animals predicted to develop a male or female fetus based on maternal testosterone divided by the number of animals actually carrying a male or female fetus (Figure 2c–d). Accuracy was defined as the number of animals with fetal gender correctly predicted divided by the total number of animals (Figure 1e; KIBUSHI et al., 2016).

## RESULTS AND DISCUSSION

Of the 21 mares in this study, 12 gave birth to female and nine to male foals. Of the determined testosterone values, four dosages were excluded from the statistical analysis because they were considered outliers by the Grubb's test. Mean ( $\pm$  SD) concentrations of maternal plasma testosterone according to the pregnancy month and sex of the foal born are summarized in table 1.

There was a statistically significant difference between plasma testosterone levels in pregnant mares carrying male fetuses compared to that of mares carrying female fetuses during the 5<sup>th</sup> and 8<sup>th</sup> months of pregnancy, and higher values were reported in mares carrying female fetuses (Figure 2). In mares carrying male fetuses, the mean value was significantly higher in the 6<sup>th</sup> month compared to the 8<sup>th</sup> ( $P < 0.05$ ; Figure 3).

Threshold values for maternal testosterone levels used to predict fetal sex were established by analyzing the ROC curves for months 5 and 8 of pregnancy (Figure 4). Values were 35.5 pg/mL and 40 pg/mL, for the 5<sup>th</sup> and 8<sup>th</sup> month of pregnancy, respectively. Mares presenting plasma testosterone equal to or higher than the threshold were predicted to be carrying female fetuses; mares with plasma testosterone below the threshold were predicted to be carrying male fetuses. Predictive values for males and females, detection rates and the accuracy of sex prediction according to maternal testosterone are described in tables 2 and 3.

(a)	Predictive value for males = number of males born ÷ number of mares predicted by plasma testosterone to be carrying male foals
(b)	Predictive value for females = number of females born ÷ number of mares predicted by plasma testosterone to be carrying female foals
(c)	Detection rate for males = number of mares predicted by plasma testosterone to be carrying male foals ÷ number of males born
(d)	Detection rate for females = number of mares predicted by plasma testosterone to be carrying female foals ÷ number of females born
(e)	Accuracy = number of animals with sex correctly predicted ÷ total number of animals

Figure 1 - Equations used for calculations of predictive values, detection rates and accuracy of fetal sex prediction in mares based on mean ( $\pm$  SD) concentrations of plasma testosterone.

The present study evaluated the levels of plasma testosterone in pregnant mares from the 5<sup>th</sup> to the 8<sup>th</sup> month of pregnancy. However, in mares carrying male fetuses, the mean value was significantly higher in the 6<sup>th</sup> month when compared to the 8<sup>th</sup> ( $P < 0.05$ ; Figure 3). These data corroborated those reported by SILBERZAHN et al. (1984), who reported maximum

values of testosterone in mares between 180 and 210 days of pregnancy. According to some authors, the variation in testosterone levels accompanies the development of fetal gonads, which, from the second month of pregnancy, are progressively invaded by steroidogenic interstitial tissue. During the 7<sup>th</sup> month, the weight of the fetal gonads is greater than that of the maternal ovaries and from the

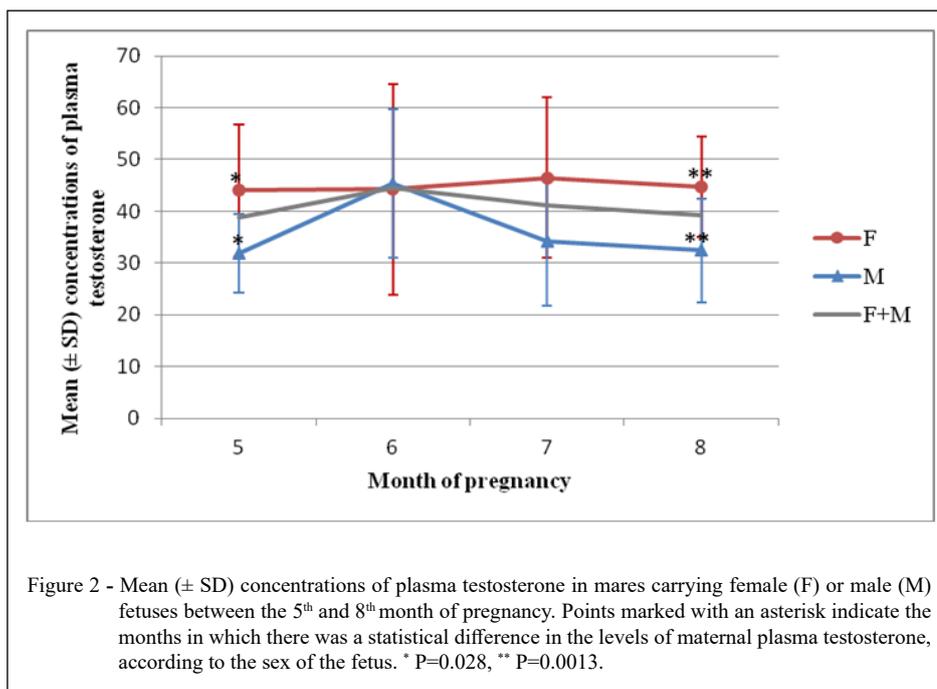


Table 1 - Mean ( $\pm$  SD) concentrations of plasma testosterone in pregnant mares, from the 5th to the 8th month of pregnancy, according to the sex of the fetus.

Month of pregnancy	Plasma testosterone (pg/mL) in mares carrying female fetuses*	Plasma testosterone (pg/mL) in mares carrying male fetuses*	P-value
5 <sup>o</sup> (n = 19)	44 $\pm$ 12.7 <sup>a</sup>	31.9 $\pm$ 7.6 <sup>Bab</sup>	0.028
6 <sup>o</sup> (n = 20)	44.2 $\pm$ 20.3 <sup>a</sup>	45.3 $\pm$ 14.3 <sup>BA</sup>	>0.05
7 <sup>o</sup> (n = 21)	46.5 $\pm$ 15.5 <sup>a</sup>	34.2 $\pm$ 12.4 <sup>aAB</sup>	>0.05
8 <sup>o</sup> (n = 20)	44.8 $\pm$ 9.7 <sup>a</sup>	32.4 $\pm$ 10 <sup>aB</sup>	0.0013

Different lower-case letters in the same line are used to indicate significance level of  $P < 0.05$ .

Different upper-case letters in the same column are used to indicate significance level of  $P < 0.05$ .

n – number of animals considered in the calculations according to the month of pregnancy (values detected as outliers were excluded).

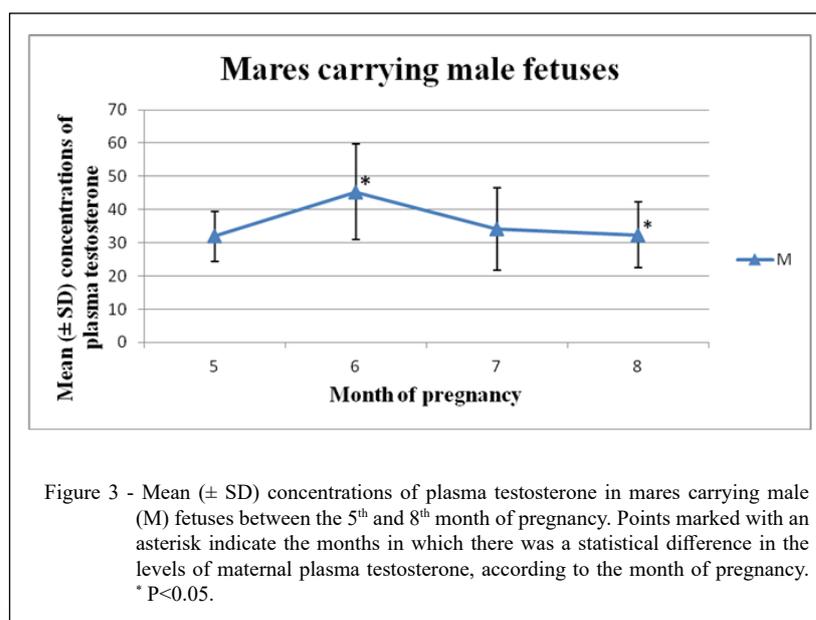
\*Mean concentrations  $\pm$  standard deviation (SD).

8<sup>th</sup> month this interstitium is progressively reabsorbed, leading to significant gonadal regression (HAY & ALLEN, 1975 *apud* SILBERZAHN et al., 1984; MERCHANT-LARIOS, 1979 *apud* SILBERZAHN et al., 1984).

SATUÉ et al. (2019) conducted a study with the objective of establishing reference values for testosterone, androstenedione and dehydroepiandrosterone concentrations in pregnant mares. These authors reported an increase in maternal testosterone during the 2<sup>nd</sup> and 3<sup>rd</sup> months of pregnancy, a plateau between the 4<sup>th</sup> and 6<sup>th</sup> months, a marked decrease from the 7<sup>th</sup> to the 9<sup>th</sup> month and a significant increase from the 10<sup>th</sup> to the last month.

The differences between the results of that study and those reported by SILBERZAHN et al. (1984) were attributed to the methodology used to measure testosterone (while SATUÉ et al. (2019). used ELISA, SILBERZAHN et al. (1984). used RIA). Despite the differences between the results, SATUÉ et al. (2019) believed that the increase in testosterone and DHEA concentrations during mid-pregnancy is of fetal origin.

Considering the possible fetal origin of plasma testosterone in pregnant mares and the studies conducted in humans, elephants and cattle where researchers reported a correlation between male fetal



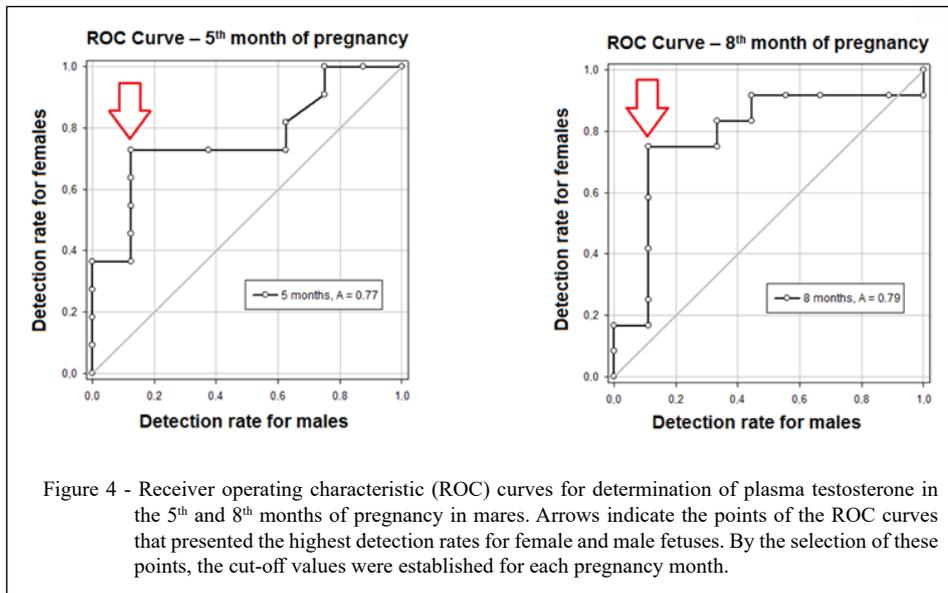


Figure 4 - Receiver operating characteristic (ROC) curves for determination of plasma testosterone in the 5<sup>th</sup> and 8<sup>th</sup> months of pregnancy in mares. Arrows indicate the points of the ROC curves that presented the highest detection rates for female and male fetuses. By the selection of these points, the cut-off values were established for each pregnancy month.

sex and higher concentrations of maternal plasma testosterone, the hypothesis of the present study is that mares gestating male fetuses would have plasma testosterone concentrations higher than those gestating female fetuses. However, results reported did not confirm this hypothesis.

Levels of maternal plasma testosterone in the present study showed higher values in mares carrying female fetuses in the 5<sup>th</sup> and 8<sup>th</sup> months than in mares carrying male fetuses. Although, significant differences were reported only in the 5<sup>th</sup> and 8<sup>th</sup> months of pregnancy, it was observed that testosterone values tended to be higher in mares carrying female fetuses (Figure 2). These data differed from those reported for humans (MEULENBERG & HOFMAN, 1991), elephants (DUER et al., 2002) and cows (KIBUSHI et al., 2016).

An exception occurred in the 6<sup>th</sup> month,

when the mean value for mares carrying male fetuses was slightly higher ( $P>0.05$ ). Although, no measurements have been performed in the last 3 months, this trend is believed to persist to the end of pregnancy and in the foal's first days of life; since circulating testosterone in neonates is higher in female foals (600–750 pg/mL and 400–500 pg/mL for 0 hours and 48 hours after birth, respectively; NAKAI et al., 2007) than in male foals (500–600 pg/mL and 200–250 pg/mL for 0 and 48 hours after birth, respectively; DHAKAL et al., 2011). Although, in the present study we were unable to identify the reasons to explain why higher testosterone concentrations were reported in mares gestating female fetuses, results of DHAKAL et al. (2011), who studied colts, compared to those by NAKAI et al. (2007), who studied fillies, suggested that female fetuses have higher circulating testosterone concentrations than

Table 2 - Calculations of predictive values, detection rates and accuracy of fetal sex prediction in mares, based on maternal plasma testosterone levels in mares in the 5<sup>th</sup> month of pregnancy.

5 <sup>th</sup> month of pregnancy (n = 19)	n	Males born	Females born	Predictive values
Mares predicted to be carrying male fetuses. Testosterone <35.5 pg/mL	10	7	3	7/10 (70%)
Mares predicted to be carrying female fetuses. Testosterone ≥35.5 pg/mL	9	1	8	8/9 (88.9%)
Detection rates		7/8 (87.5%)	8/11 (72.7%)	Accuracy 15/19 (78.9%)

Table 3 - Calculations of predictive values, detection rates and accuracy of fetal sex prediction in mares, based on maternal plasma testosterone levels in mares in the 8<sup>th</sup> month of pregnancy.

8 <sup>th</sup> month of pregnancy (n = 20)	n	Males born	Females born	Predictive values
Mares predicted to be carrying male fetuses. Testosterone <40 pg/mL	10	8	2	8/10 (80%)
Mares predicted to be carrying female fetuses. Testosterone ≥40 pg/mL	10	1	9	9/10 (90%)
Detection rates		8/9 (88.9%)	9/11 (81.8%)	Accuracy 17/20 (85%)

do male fetuses. Thus, we believed that more studies are needed to confirm or refute our findings and to seek the cause for the differences identified.

In the present experiment, significantly higher values were reported in mares carrying female fetuses during the 5<sup>th</sup> and 8<sup>th</sup> months of pregnancy. Limit values were defined for these two periods through the analysis of the ROC curve. The predictive value for diagnosis of male fetuses in the 5<sup>th</sup> month of pregnancy was 70%, which means that of the 10 mares diagnosed as carrying male fetuses by testosterone measurement (values below 35.5 pg/mL), seven were actually carrying males. The predictive value for females (values equal to or higher than 35.5 pg/mL) in 5<sup>th</sup> month of pregnancy was 88.9% (8/9). The detection rate for males in the 5<sup>th</sup> month was 87.5%, which means that of the eight mares that were actually carrying males, seven could be diagnosed as such by the hormone measurement. For females, the detection rate in the 5<sup>th</sup> month was 72.7% (8/11). These values led to a total accuracy of 78.9% for fetal sex prediction in the 5<sup>th</sup> month (15 out of 19 fetuses were correctly diagnosed; Table 2).

In the 8<sup>th</sup> month, the predictive values for male and female fetuses (limit value of 40 pg/mL) were 80% (8/10) and 88.9% (8/9), respectively. Detection rates for males and females were 88.9% (8/9) and 81.8% (9/11), respectively. The overall accuracy in the 8<sup>th</sup> month was 85% (17/20; Table 3). Results of the present study were similar to those of KIBUSHI et al. (2016), who obtained predictive values for diagnosis of fetal sex in cows (based on maternal testosterone levels) between 75% and 88.9%. Detection rates obtained by these authors were 61.5% to 93.8% and accuracy between 75.8% and 79.3%. These values varied according to the pregnancy month and breed evaluated.

In the present study, the accuracy of fetal sex prediction based on maternal plasma testosterone was

higher in the 8<sup>th</sup> month of pregnancy compared to the 5<sup>th</sup> month. Considering that fetal sexing by conventional methods is rarely performed from the 5<sup>th</sup> month, maternal plasma testosterone measurement may be an alternative for predicting fetal sex in more advanced pregnancies. Despite its inferior accuracy compared to conventional methods, this technique has the following advantages: low invasiveness, which minimizes risks to the mare and allows its application to mares of small breeds; and the possibility of predicting fetal sex in a period that is not explored by other techniques.

## CONCLUSION

It was concluded that there was a correlation between fetal sex and plasma testosterone concentrations in pregnant mares in the 5<sup>th</sup> and 8<sup>th</sup> months of pregnancy. Prediction of fetal sex based on the levels of maternal plasma testosterone can be performed in the 5<sup>th</sup> and 8<sup>th</sup> months of pregnancy with 78.9% and 85% accuracy, respectively.

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## DECLARATION OF CONFLICT OF INTERESTS

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The experiment was approved by the ethics committee on the use of animals, Sector of Agricultural Sciences of the Federal University of Paraná (protocol number 117/2016).

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