Bulgarian Journal of Veterinary Medicine (2007), **10**, No 3,185–193

DETERMINATION OF THE NUMBER OF FETUSES IN SHEEP BY MEANS OF BLOOD PROGESTERONE ASSAY AND ULTRASONOGRAPHY

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Summary

Yotov, S., 2007. Determination of the number of fetuses in sheep by means of blood progesterone assay and ultrasonography. *Bulg. J. Vet. Med.*, **10**, No 3, 185 –193.

The studies were carried out on 45 sheep during the reproductive season. A programmed artificial insemination was performed, after synchronization of the estrus with vaginal sponges Chronogest, and treatment with Folligon. Progesterone analysis and transrectal examination were accomplished on the day of insemination, 20, 40, and 60 days afterwards. It was determined that on the 60th day of pregnancy, the level of progesterone in sheep carrying two foetuses (26.9 ± 5.0 ng/mL) was higher ($p<0.01$) compared to that in sheep carrying just one foetus (20.1 ± 3.0 ng/mL). The specificity of the transrectal ultrasonography (84.2%) by the 40th day of pregnancy was higher ($p<0.05$) than that of the progesterone assay (66.5%). The summarized data showed that measuring the progesterone levels by means of ELISA on the 20th day after insemination could be used with success for diagnostics in pregnant and non-pregnant sheep. The determination of the number of foetuses in the studied sheep breeds through ultrasonography could be performed earlier (40th day), compared to that by progesterone method (60th day).

Key words: foetus number, progesterone, sheep, ultrasonography

INTRODUCTION

Determining the number of foetuses allows for planning the technological regimen of feeding and breeding, herd replacement, and to protect the sheep from toxeamia in the late stage of pregnancy (Gearhart *et al.*, 1988; Bretzlaff *et al.*, 1993). For this purpose, various methods are in use, the most commonly applied being the assay of blood progesterone in pregnant sheep and ultrasonography (Gadsby *et al.*, 1972; Kähn *et al.*, 1992).

As early as in the research by Bassett *et al.* (1969) and Robertson & Sarda (1971), it is reported that blood progesterone concentration tended to be higher in sheep carrying two fetuses. The results of Müller *et al.* (2003) showed that the number of foetuses could be determined on the 19th day of pregnancy via the progesterone concentration with 78% accuracy, while in the same period, Boscos *et al.* (2003) did not discover any significant variations in the blood progesterone in sheep carrying one or more foetuses. In their research, measuring the progesterone levels in order to determine the number of foetuses, Karen *et al.* (2006) registered a general accuracy of the method of 62% in the period 43rd–56th day after insemination , and 65.4% for the period 76th–87th day. According to Bostedt & Dedie (1996), in the earliest stage of pregnancy, the diagnostic

error was higher because of the risk of embryonic death.

Introducing ultrasonography for diagnosing pregnancy and the number of foetuses in sheep gives new possibilities for increasing the efficiency of the reproduction (Bretzlaff *et al.*, 1993). Gearhart *et al.* (1988) discovered a significant ($P<0.005$) influence of the gestation stage (0^{th}–25^{th} day) on the accuracy of the echographic method. The application of transrectal echography to determine the number of fetuses is possible, according to Kähn *et al.* (1992) even before the 20^{th} day of pregnancy, based on the clear visualization of anechoic foetus bubbles, placed one next to the other. According to Kaulfuß *et al.* (1996), however, visualization of the embryos is mostly suitable for counting them, and that is only possible after the 26^{th} day. Using the transrectal approach, and a 5 MHz linear probe, Zipper *et al.* (1997) counted the foetuses in the period 17^{th}–69^{th} day after insemination with the highest accuracy (89.1%) in the period 35^{th}–46^{th} day. Using the same technique, however, Ślósarz *et al.* (1999) registered an accuracy of 80% on the 19^{th} day, and 93% on the 25^{th} day after insemination. Karen *et al.* (2006) reported that the accuracy of the method also depends on the late embryonic and foetal death. Most authors announced the influence of the animals' breed, the stage of pregnancy, and the season on the progesterone levels in the blood of pregnant animals, and the parameters of the ultrasonographic method (Gearhart *et al.*, 1988; Müller *et al.*, 2003; Karen *et al.*, 2006).

Despite the performed research on the matter, the data on detection of the number of foetuses in the first half of pregnancy, by measuring progesterone levels and echography, are still contradictory. There is no information on this matter about sheep breeds traditionally reared in Bulgaria.

The aim of this study was to determine the possibilities for determining the number of foetuses in local sheep breeds, by measuring blood progesterone levels and by means of ultrasonography, in the first two months of pregnancy.

MATERIALS AND METHODS

Experimental animals

In this study, 45 sheep were included, 21 from the Pleven Blackhead breed, aged 1.8–4 years, weighing 45–65 kg, and 24 sheep from the Trakia Merino breed, aged 1.6–5 years, weighing 40–60 kg. The experiment was performed during the breeding season (August – September).

We also performed synchronization of the oestrus with vaginal sponges, containing 30 mg fluorogestone acetate (Chronogest, Intervet International, Netherlands), staying in the sheep's vagina for 12 days. Immediately after sponge retrieval, the animals were treated i.m. with 500 IU serum gonadotropin (Folligon, Intervet International, Netherlands).

Artificial insemination was carried out with fresh semen (0.2 cm^3, 80×10^6 mobile spermatozoa) on the 48^{th} and 56^{th} hour after sponge removal.

Progesterone (P_4) assay

Blood samples were obtained from the jugular vein on the day of insemination, (day 0) on the 20^{th}, 40^{th}, and 60^{th} day afterwards. After separating the blood serum, the samples were frozen at –20 °C until analysis. We did not obtain blood samples on the 40^{th} and 60^{th} day from sheep presenting low progesterone concentrations (<5 ng/mL) and negative ultra-

sonographic diagnosis of pregnancy on the 20th day.

The blood progesterone assay was performed with the ELISA kit (Human Gesellschaft, Biochemica Diagnostica GmbH, Germany). Cross reactivity of the test with other steroids was below 3.5% for 17-OH progesterone, corticosterone, 11-desoxycorticosterone, 11-desoxycortisol, pregnenolone, and below 0.1% for 17-β estradiol, testosterone, DHEA-S, cortisol, and cortisone. Inter- and intra-assay coefficients of variation were 15% and 20%, respectively. Minimum and maximum detectable progesterone concentrations were 0.07 ng/mL and 40 ng/mL respectively.

The threshold values of progesterone for single and twin pregnancy were determined, as well as the non-indicative values and the percentage of the non-indicative diagnoses for the different periods of research.

Ultrasonography

All sheep were ultrasonographically examined in standing position through a transrectal approach, on the 20th, 40th, and 60th day after insemination. For the ultrasonic examination, we used an Aloka SSD 500 Micrus (Tokyo, Japan, Co. Ltd) equipment with a 5 MHz linear probe, and echographic gel (Eco-supergel, Forli, Italy).

Before the echographic examination, all animals were subjected to a 12-hour fasting. After rectal insertion of the preliminarily lubricated probe, it was turned to the left, and the right under 90° angle, to find the uterus and focus the image.

The number of embryos/foetuses was determined at B and B/B working mode of the ultrasonic apparatus, with their visualization being taken into consideration during the early stage (20th day), and in following tests, the heart activity and spontaneous foetal movement indicators were also taken into account.

After establishing the final data from the birth, for both methods we determined accurate positive diagnoses (two foetuses), inaccurate positive diagnoses (one foetus), accurate negative diagnoses (one foetus), and inaccurate negative diagnoses (two foetuses). Based on that information, we calculated the sensitivity, accuracy, positive predicted values, negative predicted values, and general accuracy, by the method described by Martin *et al.* (1987).

Statistical analysis

All results were statistically processed with the computer programme StatSoft (Microsoft Corp. 1984-2000 Inc.) using the ANOVA, LSD-test, correlation analysis, and Other significant test options to calculate the levels of significance in the differences between two ratios (%).

RESULTS

We did not establish any significant variations in the values of progesterone in pregnant sheep of the different breeds for the four experimental periods. On the 20th day after insemination, the concentrations of progesterone in pregnant Pleven Blackhead and Trakia Merino sheep were 11.2 ± 3.6 ng/mL and 12.3 ± 4.1 ng/mL respectively, significantly higher ($P<0.01$) than the values for the same interval in inseminated but non-fertilized sheep (3.9 ± 1.1 ng/mL and 3 ± 0.9 ng/mL respectively). Based on this information, 34 sheep were determined to be pregnant, and 11 as non-pregnant.

A strong positive correlation ($r=0.98$, $P<0.01$) between the stage of pregnancy and the concentrations of P_4 was estab-

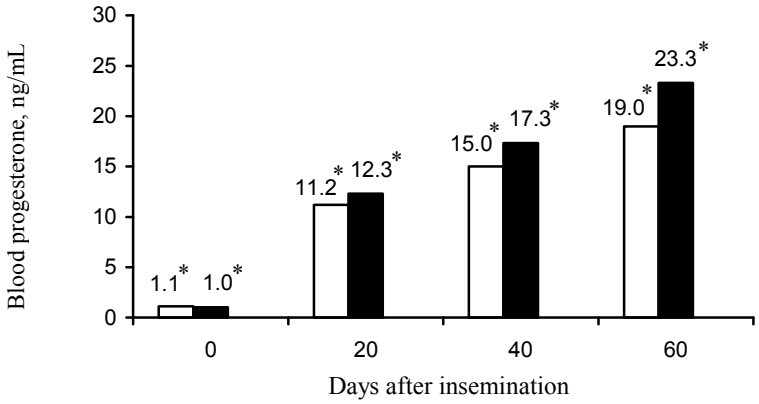

Fig. 1. Progesterone concentrations in pregnant sheep by the day of insemination (day 0), and on the 20th, 40th and 60th days of pregnancy. (■) Pleven Blackhead, (□) Trakia Merino sheep. * P<0.01 vs sheep from the same breed in each time periods.

lished in 32 animals, and the variations in the values of progesterone between the separate periods were significant at P<0.01 (Fig. 1). For two Trakia Merino sheep, however, a decrease in the levels of progesterone from 12.3 ng/mL to 1.7 ng/mL (on post insemination day 20) and from 16.9 ng/mL to 5.2 ng/mL (on post insemination day 60) was detected.

After reading the birth data from the 34 positive sheep, 32 diagnoses were confirmed. The sheep were divided into two groups – with single and twin pregnancy. From the total of 32 confirmed births, 20 sheep exhibited single and 12 exhibited twin pregnancy. No triplets were registered.

When comparing the values of P_4 for both groups on the 20th and 40th day of pregnancy, no significant differences were found. On the 60th day, the progesterone levels (26.9 ± 5.0 ng/mL) of the animals with twin-foetus pregnancy were significantly (P<0.01) higher than the levels (20.1 ± 3.0 ng/mL) of animals with single-foetus pregnancy (Table 1). The determined threshold values of P_4 during the separate periods of research were <8.8, <13.2, and <21.9 ng/mL for sheep with one foetus, and >15.1, >19.1, and >23.1 ng/mL for sheep with twin foetuses. The highest percentage (29.5%) non-indicative diagnoses was established on the 20th day, while there were no such on the 60th day. The method's parameters – sensitivity, accuracy, positive predicted and negative predicted values, and total accuracy were the highest on the 60th day of pregnancy – 78.5%, 88.8%, 88.7%, 84.2%, and 84.4% respectively (Table 2).

We did not discover any significant differences in the peripheral concentrations of progesterone in sheep carrying foetuses of different genders.

Using ultrasonography on the 20th day after insemination, from a total of 45 sheep (100%), 27 (60%) were diagnosed as pregnant (positive diagnosis), and 18 (40%) were diagnosed as non-pregnant (negative diagnosis). In the next two peri-

Table 1. Progesterone concentrations (ng/mL) in single and twin pregnancy and parameters of progesterone assay on the 20th, 40th and 60th days of pregnancy in Pleven Blackhead and Trakia Merino sheep

Items	Days of pregnancy		
	20	40	60
Progesterone levels in single pregnancy (n=20; mean ± SD)	11.1± 3.8	15.2± 4.1	20.1± 3.0
Progesterone levels in twin pregnancy (n=12; mean ± SD)	12.3 ±3.5	18.2 ±5.0	26.9 ±5.0 [b]
Threshold values in single pregnancy (ng/mL)	< 8.8	<13.2	<21.9
Threshold values in twin pregnancy (ng/mL)	>15.1	>19.1	>23.1
Non-indicative values (%)	8.9–15	13–19	22–23
Non-indicative diagnoses (%)	29.5	17.6	0

[b] p<0.01 vs single pregnancy.

Table 2. Parameters of progesterone (P_4) assay and ultrasonography on the 20th, 40th and 60th days of pregnancy in Pleven Blackhead and Trakia Merino sheep

Parameters	P4 (ELISA) – days of pregnancy			Ultrasonography – days of pregnancy		
	20	40	60	20	40	60
Sensitivity, %	71.4 [b]	72.7	78.5	30.0	69.2	80.0
Specificity, %	83.3	66.5 [a]	88.8	88.2	84.2	80.0
Positive predicted values, %	83.3	66.6	88.7 [a]	66.0	75.0	66.7
Negative predicted values, %	71.4	72.7	84.2	68.2	80.0	80.0
Total accuracy, %	76.9	70.0	84.4	66.6	78.7	75.0

[a] P<0.05; [b] P<0.01 vs ultrasonography.

ods of study, the positive diagnoses were 32 (71%), and the negative – 13 (29%). The highest sensitivity (80%) was established on the 60th day, accuracy (88.2%) – on the 20th day, and positively, negatively predicted values and total accuracy (75%, 80%, and 78.7% respectively) – on the 40th day of pregnancy (Table 2).

Comparing the two methods, we determined a significantly higher (P<0.01) sensitivity (71.4%) of the progesterone assay, than that of ultrasonography (30%) on the 20th day. On the 40th day, the accuracy of the latter (84.2%) was higher than the accuracy of the progesterone assay (66.5%) (P<0.05). On the 60th day, the results showed a significant (P<0.05) difference between the positively predicted diagnoses by ELISA (88.7%), and transrectal ultrasonography (66.7%).

DISCUSSION

In their studies, Cahill *et al.* (1981) and Bratlewski *et al.* (1999), reported differences in the peripheral levels of proges-

terone in sheep of different breeds. Our results, however, do not show any significant differences in the levels of progesterone for the studied breeds of sheep, during the first two months of pregnancy. This suggests that the used method is suitable for progesterone screening in mixed herds of the examined sheep. The finding of significant ($P<0.01$) differences in the concentrations of the studied hormone between pregnant and non-pregnant sheep matches the data of Boscos *et al.* (2003), who registered that fact on the 19th day after insemination. The positive correlation between the levels of progesterone and the gestation stage confirms the statement by Mukasa-Mugerwa & Viviani (1992) that its values on the 40th day of pregnancy are higher than the peak values during the lutheal stage of the sexual cycle, which is due to an extraovarian source of progesterone (placental synthesis). According to Gray *et al.* (2001) and Spencer *et al.* (2004) between the 15th and 50th day of pregnancy, the endometrial glands undergo a strong hyperplasia and hypertrophy by synthesizing, secreting, and transporting various enzymes, cytokines, lymphokines, hormones, transport proteins and other substances. In support of this notion are the established significantly higher ($P<0.01$) progesterone concentrations in pregnant vs non-pregnant sheep in different periods of the study for both sheep breeds. The decrease of progesterone in two of the sheep was, according to us, an indicator for late embryonic death, which, according to Bostedt & Dedie (1996), reaches 15% in this gestation period. This was confirmed by ultrasonography on the 40th and 60th day. While on the 20th day, we found increased uterine lumen and amniotic fluid, this could not be found in later examinations.

The determined lack of significant differences in the peripheral blood levels of progesterone between sheep with one and two foetuses on the 20th and 40th gestation day is in contrast with the results by Müller *et al.* (2003), which can distinguish between single-foetus and multi-foetus pregnancy on the 19th day after insemination in sheep of the German Blackhead x Long-wool Merino breed. In support of our observations, however, are the results by Boscos *et al.* (2003) for the Chios, Berichon, and Safakia breeds, evidencing an absence of significant variations in the same period. According to us, these controversies are probably caused by the breed peculiarities of the animals, and the fact that, during the early stage of pregnancy in sheep, the yellow body is the primary source of progesterone. This is confirmed by the studies of Davies *et al.* (2006), which found a positive correlation between the levels of progesterone and the surface of the yellow body in sheep breeds with different ovulation values.

The results from the 60-th day showed that it is possible to determine the number of foetuses for the examined breeds of sheep. They confirmed the hypothesis by Goel & Agrawal (1989) for faster utilization of progesterone in multiple pregnancy. Unlike Chauhan & Waziri (1991) and Kalkan *et al.* (1996), who reported significant differences in the progesterone levels between sheep with one or more fetuses during the second half of pregnancy, we determined significant ($P<0.01$) differences as early as the 60th day. The large percentage (29.5% and 17.6%) of non-indicative diagnoses on the 20th and 40th days after insemination could be explained with the variations in progesterone concentrations between the different animals and embryonic death in this gestation stage. According to Schaetz & Leidl

(1983), one of the reasons for that is the partial embryonic loss – death of one of the embryos in sheep with multiple pregnancy, which influences the method's parameters.

Ultrasonography on the 20th day showed that, in this stage of pregnancy, the diagnostic error was high. This can be explained with the impossibility to detect changes in the uterus in all animals, as well as the low sensitivity of echography, caused by unconfirmed diagnoses, due to embryonic death and the inaccurate criteria for establishing a positive diagnosis. This supports the strong influence of the stage of pregnancy on the parameters of the echographic method up to the 25th day of pregnancy, established by Gearhart *et al.* (1988).

Later examinations on the 40th and 60th days confirmed this, and matched the thesis of Kaulfuß *et al.* (1996) for using accurate criteria in the diagnostics of pregnancy and determining the number of foetuses.

The results derived from comparing the parameters of both methods showed that, on the 20th day of pregnancy, the determination of progesterone was more reliable than echography, in order to prove pregnancy, and the number of foetuses. This was also confirmed by the established significant ($P<0.01$) difference in the levels of the hormone, and the sensitivity percentage between pregnant and non-pregnant animals. The data about the accuracy of ultrasonography did not confirm the results of Ślósarz *et al.* (1999), but match those of Zipper *et al.* (1997). The observed higher ($P<0.05$) accuracy of echography on the 40th day supports this, and is due to the precise criterion of counting the foetuses - visualizations of embryos with heart activity. The higher percentage ($P<0.05$) of positively predicted values by the progesterone method on the 60th day shows that it was suitable to determine the number of foetuses in examined sheep breeds. The lack of non-indicative diagnoses is also supporting this opinion. The lower percentage for echography is caused by the diagnostic error in proving multiple pregnancy. Probably, it is due to the used approach at that stage of pregnancy, with the uterus being dislocated low in the abdomen in some cases, which obstructs the correct diagnostics of the number of foetuses. This confirms the statement by White *et al.* (1984) about the reasons for diagnostic error.

Even though we did not find any significant differences between progesterone method and ultrasonography with regard to negatively predicted values and the total accuracy, the established percentages for these parameters for the progesterone method on the 60th day, and the echography on the 40th day were close to the results by Karen *et al.* (2006) and Zipper *et al.* (1997).

After analysis of the results, we can conclude that assaying the progesterone levels via ELISA on the 20th day after insemination can be successfully used for diagnostics of pregnant and non-pregnant sheep. Determining the number of foetuses in the examined sheep breeds by ultrasonography can be performed earlier (40th day), compared to the progesterone method (60th day).

REFERENCES

Bassett, J., J. T. Oxborrow, I. D. Smith & G. D. Thorburn, 1969. The concentration of progesterone in peripheral plasma of the pregnant ewe. *Endocrinology,* **45,** 499.

Bartlewski, P. M., A. P. Beard & N. C. Rawlings, 1999. Ovarian function in ewes

at the onset of the breeding season. *Animal Reproduction Science,* **57,** 67–88.

Boscos, C. M., F. C. Samartzi, A. G. Lymberopoulos, A. Stefanakis & S. Belibasaki, 2003. Assessment of progesterone concentration using enzymeimmunoassay, for early pregnancy diagnosis in sheep and goats. *Reproduction in Domestic Animals*, **38,** 170–174.

Bostedt, H. & K. Dedie, 1996. Embryonic death. In: *Sheep and Goats Diseases,* Eugen Ulmer, Stuttgart, pp. 463–480.

Bretzlaff, K., J. Edwards, D. Forrest & L. Nuti, 1993. Ultrasonographic determination of pregnancy in small ruminants. *Veterinary Medicine,* **88,** 12–24.

Cahill, L. P., J. Saumande, J. P. Ravault, M. Blanc, J. Thimonier, J. C. Mariana & P. Mauleon, 1981. Hormonal and follicular relationships in ewes of high and low ovulation rates. *Journal of Reproduction and Fertility,* **62,** 141–150.

Chauhan, F. S. & M. A. Waziri, 1991. Evaluation of rectal-abdominal palpation technique and hormonal diagnosis of pregnancy in small ruminants. *Indian Journal of Animal Reproduction,* **12,** 63–67.

Davies, K. L., P. M. Bartlewski, R. L. Pierson & N. C. Rawlings, 2006. Computer assisted image analyses of corpora lutea in relation to peripheral concentrations of progesterone: A comparison between breeds of sheep with different ovulation rates. *Animal Reproduction Science,* **96,** No 1–2, 165–175.

Gadsby, J. E., R. B. Heap, D. G. Powell & D. E. Walters, 1972. Diagnosis of pregnancy and of number of fetuses in sheep from plasma progesterone concentrations. *Veterinary Record,* **90**, 339–342.

Gearhart, M. A., W. E. Wingfield, A. P. Knight, J. A. Smith, D. A. Daraatz, J. A. Boon & C. A. Stokes, 1988. Real-time ultrasonography for determining pregnancy status and viable fetal numbers in ewes. *Theriogenology,* **30,** 323–337.

Goel, A. K. & K. P. Agrawal, 1989. Pregnancy diagnosis in sheep. *Indian Journal of Animal Science,* **59,** 974–976.

Gray, C. A., F. F. Bartol, B.J. Tarleton, A. A. Wiley, G. A. Johnson, F. W. Bazer & T. E. Spencer, 2001. Developmental biology of uterine glands. *Biology of Reproduction,* **65,** 1311–1323.

Kähn, W., B. Kähn, A. Richter, J. Schulz & M. Wolf, 1992. Sonography during the pregnancy of sheep. Fetometry for determination of the stage of gestation and prediction of the time of parturition. *Deutsche tierärztliche Woschenschrift,* **99,** 449–452.

Kalkan, C., H. Cetin, E. Kaygusuzoglu, B. Yilmaz, M. Ciftci, H. Yildiz, A. Yildiz, H. Deveci, A. M. Apaydin & H. Ocal, 1996. An investigation on plasma progesterone levels during pregnancy and parturition in the Ivesi sheep. *Acta Veterinaria Hungarica,* **44,** 335–340.

Karen, A., B. El Amiri, J.-F. Beckers, J. Sulon, A. M. A. Taverne & O. Szenci, 2006. Comparison of accuracy of transabdominal ultrasonography, progesterone and pregnancy-associated glycoproteins tests for discrimination between single and multiple pregnancy in sheep. *Theriogenology,* **66,** No 2, 314–322.

Kaulfuß, K. H., K. Uhlich, S. Brabant, K. Blume & K. Strittmatter, 1996. Real-time ultrasonic examination of fetal growth of sheep between day 20 and day 50 of gestation. *Tierärztliche Praxis,* **24,** 443– 452.

Mukasa-Mugerwa, E. & P. Viviani, 1992. Progesterone concentrations in peripheral plasma of Menzsheep during gestation and parturition. *Small Ruminant Rescue,* **8,** 47–53.

Martin, S. W., A. H. Meek & P. Willeberg, 1987. In: *Veterinary Epidemiology, Principles and Methods.* Iowa State University Press, Ames, Iowa, pp. 63–71.

Müller, T., H. Schubert & M. Schwab, 2003. Early prediction of fetal number in sheep based on peripheral plasma progesterone concentrations and season of the year. *Veterinary Record,* **1,** 137–138.

Robertson, H. A. & I. R. Sarda, 1971. A very early pregnancy test for mammals: Its application to the cow, ewe and sow. *Journal of Endocrinology,* **49,** 407–419.

Schaetz, F. & W. Leidl, 1983. Pregnancy diagnosis in sheep. In: *Reproductive Disturbances in Domestic Animals*, Enke, Stuttgart, pp. 437–440.

Ślósarz P., R. Steppa & A. Gadek, 1999. Proba wykorzystania ultrasonografii transrektalnej do wczesnej diagnostyki ciaży u owiec. *Medycyna Weterynaryjna*, **55**, 686–688.

Spencer, T. E., R. C. Burghardt, G.A. Johnson & F.W. Bazer, 2004. Conceptus signals for establishment and maintenance of pregnancy. *Animal Reproduction Science,* **82–83,** 537–550.

White, I. R., A. J. F. Russel & D. G. Fowler, 1984. Real-time ultrasonic scanning in the diagnosis of pregnancy and the determination of fetal numbers in sheep. *Veterinary Record,* **115,** 140–143.

Zipper, N., K. H. Kaulfuss, J. May & K. Elze, 1997. Die ultrasonographische Trächtigkeitsdiagnostik (B-Mode) beim Schaf. Teil 3: Untersuchungen zür Erfassung der Anzahl von Embryonen und Feten. *Tierärztliche Praxis,* **25,** 212–222.

Paper received 05.02.2007; accepted for publication 03.07.2007

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