BIODIVERSITAS
Volume 23, Number 11, November 2022
Pages: 6056-6061

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d231161

Urine biochemical profile analysis and transabdominal ultrasonography for pregnancy detection in the endangered lowland anoa (*Bubalus depressicornis*)

ANOM BOWOLAKSONO[1,2,♥], ANITA MAYASARI[2], AYU MULIA SUNDARI[1], ADVEN TRI JOY SIMAMORA[2], RAHMA SURYANINGSIH[2], ADY SURYAWAN[2], ABINAWANTO[1], LUTHFIRALDA SJAHFIRDI[1], AFIFA HASNA[2], JATNA SUPRIATNA[1], RANDALL C. KYES[3]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia. Jl. Prof. Dr. Sudjono D. Pusponegoro, Kampus UI, Depok 16424, West Java, Indonesia. Tel./fax.: +62-21-7872610, ♥email: alaksono@sci.ui.ac.id
[2]Environment and Forestry Research and Development Institute of Manado. Jl. Adipura, Kimia Atas, Manado 95259, North Sulawesi, Indonesia
[3]Departments of Psychology, Global Health, and Anthropology, Center for Global Field Study, Washington National Primate Research Center, University of Washington. Seattle, Washington 98195, USA

Manuscript received: 14 August 2022. Revision accepted: 22 November 2022.

Abstract. *Bowolaksono A, Mayasari A, Sundari AM, Simamora ATJ, Suryaningsih R, Suryawan A, Simamora ATJ, Abinawanto, Sjahfirdi L, Hasna A, Supriatna J, Kyes RC. 2022. Urine biochemical profile analysis and transabdominal ultrasonography for pregnancy detection in the endangered lowland anoa (*Bubalus depressicornis*). Biodiversitas 23: 6056-6061.* The population of lowland anoas (*Bubalus depressicornis*) has decreased significantly due to illegal hunting and habitat loss. As such, the urgency in developing successful ex-situ conservation programs cannot be overstated. Ex-situ breeding programs of anoas, however, are hindered by difficulties in early pregnancy detection. The failure to detect pregnancy at an early stage often results in miscarriage due to repeated breeding attempts of unconfirmed pregnant females. Moreover, a frequent false heat during 5 to 6 months of pregnancy further complicates successful breeding programs due to the remating of pregnant females that often leads to spontaneous abortion. Therefore, early detection techniques are needed to improve breeding success. The present study evaluated the use of urine biochemical profiles as a minimally invasive approach for early pregnancy detection and transabdominal ultrasonography as a confirmation tool of false heat in four lowland anoas. Statistical analysis showed that glucose and protein levels were significantly elevated during pregnancy beginning day 0 of post-mating ($p<0.05$) and consistently higher compared to the non-pregnant period. Meanwhile, transabdominal ultrasonography successfully identified organ structures and development by the 155th, 176th, 180th, and 191st days of pregnancy. The present study suggests that urine biochemical data provide a promising, minimally invasive approach to early pregnancy detection in anoas. Incorporating urine data and ultrasonography can significantly help to maintain pregnancy by validating false heat.

Keywords: Anoa, *Bubalus depressicornis*, pregnancy detection

INTRODUCTION

The lowland anoa (*Bubalus depressicornis*) is one of Indonesia's flagship species and endemic to Sulawesi Island. Despite its standing among Indonesia's unique mammalian biodiversity, the future of the lowland anoa population is at risk. With an estimated population of less than 2500 mature individuals, the lowland anoa is currently categorized as Endangered by the International Union for Conservation of Nature (IUCN) Red List (Burton et al. 2016) and classified as Appendix 1 by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The dramatic decline in the anoa population is attributed in large part to anthropogenic activity such as hunting and habitat loss (Burton et al. 2016). Given the ongoing decline of the anoa population in the wild, as much as 20% per year, the need for successful ex-situ conservation programs is crucial to prevent anoas from extinction (Burton et al. 2016). However, reproduction rates in ex-situ conservation facilities are far from sustainable since early pregnancy detection often fails, which in turn leads to spontaneous abortion due to repeated breeding (Burton et al. 2005; Burton et al. 2016). Additionally, a false heat, the appearance of estrous behavior during pregnancy (EBP), at the 5-to-6-month period of post-mating, is another obstacle that is often mistakenly recognized as estrous and results in remating of anoa (Dijkhuizen and van Eerdenburg 1997). Therefore, an early pregnancy detection method and a tool for validating EBP are urgent priorities for further investigation.

Analyzing the biochemical compounds contained in urine is one of the emerging approaches for early pregnancy detection in animals. Studies have shown that pregnancy in animals is closely associated with elevated minerals, hormones, and other substances in urine (Khan et al. 2015; Rawat et al. 2016). For example, Khan et al. (2015) reported that both glucose and protein levels in the urine of pregnant dairy cattle showed an increase. Similarly, Katiyar et al. (2015) observed that both substances also were higher in pregnant buffaloes compared to controls (Katiyar et al. 2015). These pregnancy-related changes, however, have not yet been reported in anoas.

For EBP validation, ultrasonography is thought to be the most reliable approach as it can show the appearance of

fetal development. Reports suggest that transrectal ultrasonography, a type of ultrasonography carried out by placing probes into the rectum, manages to provide accurate examination and rapid assessment without side effects (Romano and Christians 2008; Petrujkić et al. 2016) in a range of animal species, including domestic animals, primates, and other wildlife (Acosta et al. 2005; Miranda et al. 2018; Dejneka et al. 2020). Studies have shown that transrectal ultrasonography managed to show fetal imaging on the 26th and 37th day in buffalos, thus signifying its value in pregnancy evaluation (Ingawale et al. 2012; Naikoo et al. 2013). Transrectal ultrasonography, however, is an invasive procedure that requires the insertion of an ultrasonography probe into the animal's rectum. Such a procedure presents potential challenges and risks for application with the anoa, given the animal's aggressive nature (Dijkhuizen and van Eerdenburg 1997; Medan and Abd 2010).

An alternative to transrectal ultrasonography is transabdominal ultrasonography which involves a minimally invasive procedure that shows a cross-sectional presentation of the internal organs. It utilizes various acoustic impedance by placing a transducer externally to the abdominal wall. It is considered a more suitable scanning method that can be conducted on an animal in a standing position without the stress of anesthesia (Kim 2015; Kurzweil and Martin 2020). Given that the use of transabdominal ultrasonography has not yet been reported in anoas (Jones et al. 2016; Schmid et al. 2016), the aim of the current study was to evaluate the reliability of urine biochemical profiles and transabdominal ultrasonography as a minimal invasive method for early pregnancy detection and EBP validation, respectively, in anoas.

MATERIALS AND METHODS

Study site

The study was conducted between January 2017 and January 2020 at the Anoa Breeding Center Manado, located at the Environment and Forestry Research and Development Institute of Manado, North Sulawesi, Indonesia. Over the course of the study period, a total of seven anoas were present at the breeding center, including three adult males and four adult females. All four adult females were evaluated in this study; they were identified as anoa #1, anoa #2, anoa #3, and anoa #4 (Figure 1A-1D; Table 1). All procedures were approved by the Faculty of Medicine, Universitas Indonesia Ethics Committee, and the Anoa Breeding Center Manado.

Data collection

The evaluation period for each of the four female anoas began when they displayed signs of estrous (based on morphological and behavioral criteria) (Mayasari et al. 2018). When estrous was confirmed, the anoa was subsequently mated three times over a one-day period. For the urine biochemical profile analysis, fresh urine was collected from each female beginning on the day of mating and then every 7th day until the 28th day of post-mating. Urine collection was carried out between 08.00-11.00 WITA (Central Indonesian Time, UTC + 08:00). Urine was collected directly at the time of urination in plastic containers attached to an extension pole held by the staff. In cases when this collection technique was not possible, fresh urine was collected from the cage floor immediately the following urination using a disposable syringe. The urine was then filtered using filter paper into a 10 mL tube and stored in a freezer at-10℃ for subsequent analysis.

Figure 1. Four female anoas evaluated in the present study: A. Anoa #1; B. Anoa #2; C. Anoa #3; D. Anoa #4. The evaluation began on each anoa when signs of estrous appeared according to morphological and behavioral characteristics. The anoa was then mated three times over a one-day period following estrous confirmation

Table 1. Sample characteristics

Sample	Date of birth	Origin	Generation	Breeding history
Anoa #1	est. 2009	Palu, Central Sulawesi	F0	2015 (Not survived) 2017 (Male, survived) 2018 (Female, survived) 2020 (Not survived)
Anoa #2	est. 2010	Palu, Central Sulawesi	F0	2016 (Not survived) 2017 (Survived)
Anoa #3	est. 2013	Gorontalo	F0	2017 (Abortus) 2019 (Abortus)
Anoa #4	est. 2009	Palu, Central Sulawesi	F0	2019 (Not survived)

Figure 2. Ultrasonography examination. A veterinarian and an assistant perform an ultrasonography on an anoa standing in a cattle restrain cage. The ultrasonography involved the use of a convex-type abdominal ultrasonography at 2-6 MHz using 95% alcohol as a media transducer

Protein and glucose levels were determined using Total Protein Kit (Sigma-Aldrich, St. Louis 63103, USA) and Glucose Assay Kit (Sigma-Aldrich, St. Louis 63103, USA), respectively, according to the manufacturer's protocol. To measure protein, 50 µL of the urine sample was reacted with 2.5 mL of the protein assay solution, then the absorbance (A) of Standard ($A_{Standard}$) and Tests (A_{Test}) versus the Blank was read by spectrophotometer at 595 nm wavelength (Thermo Fisher Scientific, Wilmington 19810, USA). Protein concentrations were then calculated as (A_{Test}) × concentration of standard/($A_{Standard}$). For the glucose measurement, after the standard construction, 50 µL of the master reaction mix was added to 50 µL of the urine sample and positive control. Glucose concentration was then determined by spectrophotometer at 570 nm after 30 minutes of incubation.

For EBP evaluation, transabdominal ultrasonography examinations were conducted on each female beginning on the 21st day, the end of the estrous cycle, and then continued once every month for a period of eight months. Starting at 140 days, examinations were conducted twice a month to avoid delayed detection. To perform the procedure, anoas were directed into an 80x120x100 cm-sized cattle restrain cage where the abdominal hair was trimmed (Figure 2). Convex-type abdominal ultrasonography at 2-6 MHz utilizing 95% alcohol as a media transducer was used and the probe was placed in a transverse/sagittal position on the cranial pubis, rotated around the abdomen and uterus.

Data analysis

The urine biochemical profiles were analyzed using a T-test at a 95% confidence level. Transabdominal ultrasonography results were analyzed qualitatively, and interpreted by a veterinarian according to Mannion et al. (2006) and Ali and Hayder (2007).

RESULTS AND DISCUSSION

Biochemical analysis of the urine samples for all four females showed elevated levels of protein and glucose during pregnancy compared to the non-pregnant period (Figure 3). Further, the T-test results showed that these elevations were significantly different (Table 1). In terms of ultrasonography, successful ultrasonography imaging was obtained from all four anoas evaluated. For anoa #1, the ultrasonography image on the 191st day after mating showed the presence of the thoracic bone, fetal spine, head, heart, and complete lungs in the anterior longitudinal orientation (Figure 4A). In anoa #2, structures including heart, lung, and thoracic bone were observed on the 180th day after mating (Figure 4B), while the heart and lung were seen in anoa #3 on the 155th day after mating (Figure 4C). For anoa #4, ultrasound imaging on the 176th day after mating showed the presence of the heart (4D). When examining ultrasonography images on the 21st day after mating, no conclusive evidence of pregnancy could be observed for any of the anoas. Specifically, the presence of a gestational sac, typically the first sign of pregnancy, was not observed in the 21st-day image of all anoas (Figure 5A-5D).

Table 1. Urine biochemical results

Periods	Protein mean ± SD (mg/dL)	Glucose mean ± SD (mg/dL)
Pregnancy	1.93 ± 0.35	0.79 ± 0.11
Non-pregnant	1.53 ± 0.10	0.60 ± 0.24

Note: P-values were determined by a Student's T-test. Protein: Tests for differences had P-values 0.02 (t: 6.109) Glucose: Tests for differences had P-values 0.01 (t: 4.361)

Discussion

To our knowledge, this is the first study to investigate the potential use of urine profiles, as well as the characterization of transabdominal ultrasonography, for pregnancy detection and EBP evaluation in anoa. These results suggest that together, these two techniques provide a novel, minimally invasive approach for pregnancy detection and maintenance in anoas, thus aiding in the ex-situ reproductive management and conservation of anoas.

For early pregnancy detection, our results showed that protein and glucose levels were significantly higher in anoa during the pregnancy period, similar to the findings reported by Katiyar et al. (2015) with buffalo. Their study revealed that pregnancy was signaled by the elevation of total protein and glucose that resulted from the surge of glomerular filtration rate (Katiyar et al. 2015). Protein increase was considered to be associated with the increase in the glomerular filtration rate and renal plasma flow due to the fetal waste process (Katiyar et al. 2015; Petrujkić et al. 2016). Meanwhile, glucose elevation may be due to the increase in energy demand to support embryo development during pregnancy (Katiyar et al. 2015). In contrast to limited reports in animals, studies with humans have confirmed that glycosuria is common among pregnant women due to increased glomerular filtration rate (Alto 2005). The lowering of the renal threshold during pregnancy led to the presence of glucose in the urine despite normal blood glucose (Alto 2005). Further, reports suggest that low glucose is related to reproductive function disturbance, infertility, fetal death, and folliculogenesis failure (Persson and Hansson 1993; Johnson 2008). Therefore, urine biochemical profiles could be utilized as an alternative parameter for early pregnancy detection in anoas.

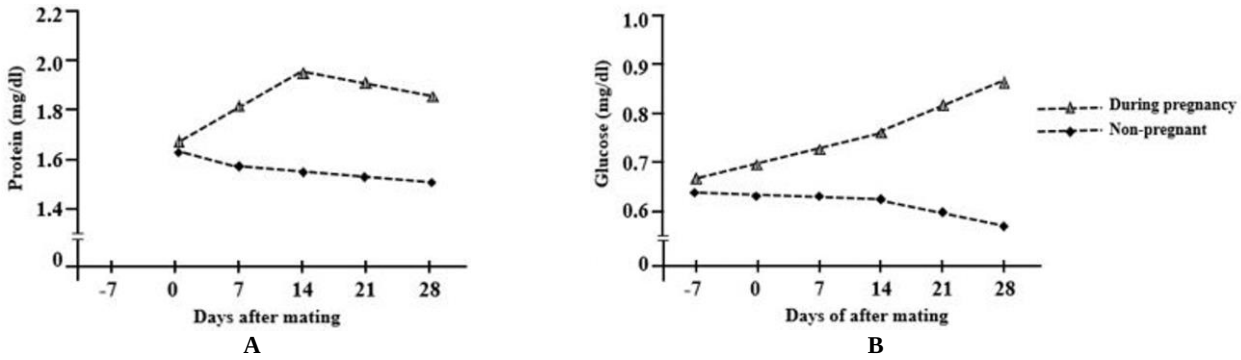

Figure 3. Daily changes in protein (A) and glucose (B) of anoas during pregnancy and non-pregnant on the 0 days until 28 days post-mating. Urine samples were collected every 7th day

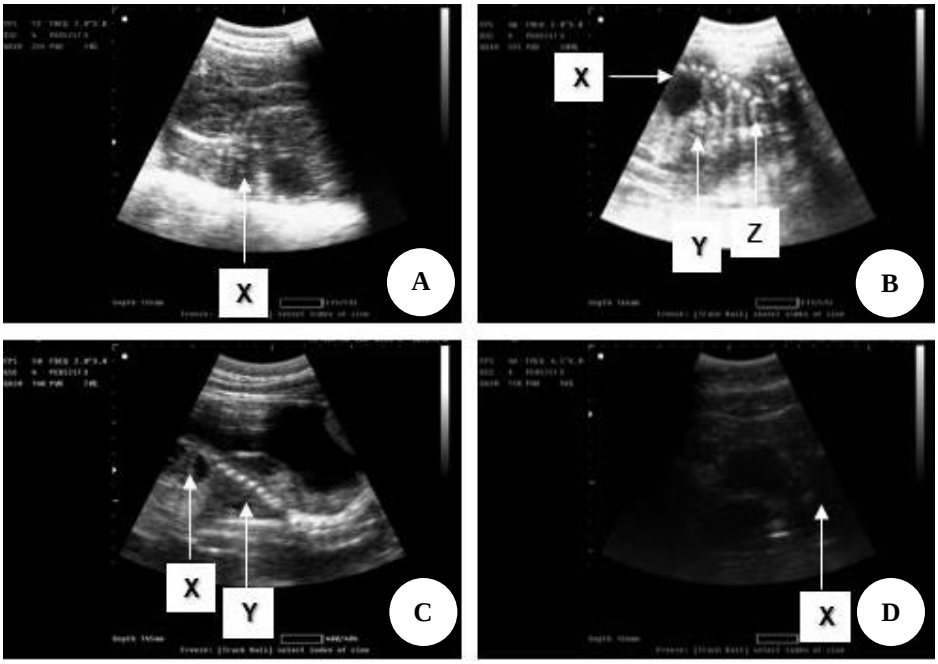

Figure 4. The ultrasonography images of: A. Anoa #1 on the 191st day of gestation (X: thoracic bone, fetal position seen in the anterior longitudinal presentation); B. Anoa #2 on the 180th day of gestation (X: heart; Y: lung; Z: thoracic bone); C. Anoa #3 on 155th day of gestation (X: heart; Y: lung); D. Anoa #4 on 176th day of gestation (X: heart). Results were obtained by convex-type abdominal ultrasonography at 2-6 MHz using 95% alcohol as a media transducer in the transversal/sagittal plane

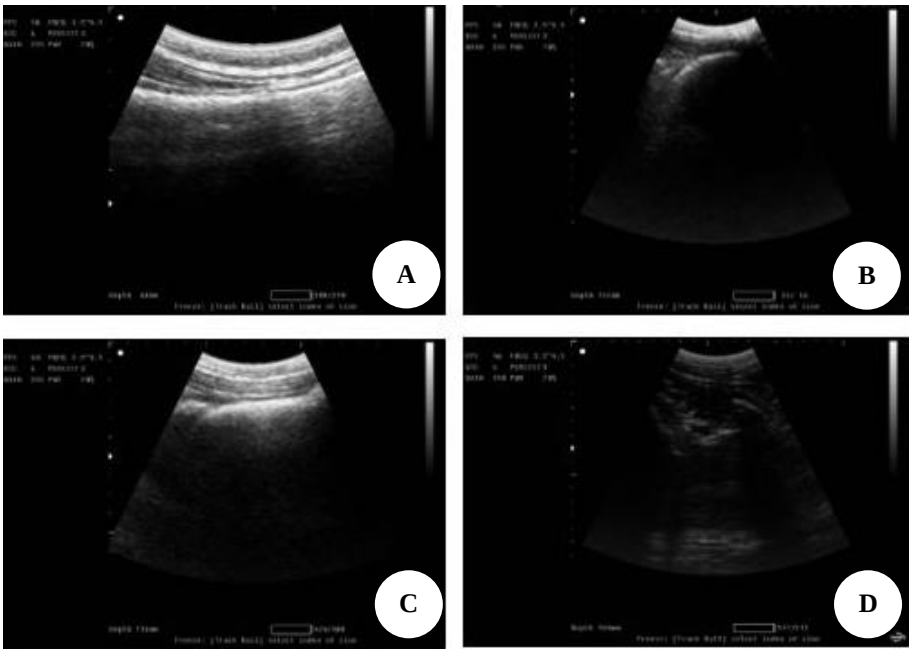

Figure 5. The ultrasonography images on the 21st day after mating of: A. Anoa #1; B. Anoa #2; C. Anoa #3; D. Anoa #4 obtained by convex-type abdominal ultrasonography at 2-6 MHz using 95% alcohol as a media transducer in the transversal/sagittal plane. The gestational sac is not visible

In terms of EBP validation, our results showed that the transabdominal method had 100% accuracy in detecting fetal development, with the earliest confirmation on the 191st day. The competence of transabdominal ultrasonography to display fetal images during these periods is essential to prevent pregnant females from unintentionally repeated breeding. Based on our experience, another challenge of pregnancy detection in anoas is that pregnant individuals often show signs of estrous at 5 or 6 months of pregnancy which can confuse animal care staff. Therefore, transabdominal ultrasonography could be utilized as a confirmatory test when the signs of EBP appear. Additionally, as parturition is sometimes hindered by abnormal fetal development and position, transabdominal ultrasonography also could be used to monitor the fetal dynamic. This, in turn, could provide input for parturition process decisions, which in some cases, necessitate a cesarean procedure due to dystocia (difficult labor) (Hohnholz et al. 2019; Dejneka et al. 2020). This is very important to prevent birth loss. Moreover, we found that the use of 95% alcohol was more reliable as a media transducer than a gel, as we were able to obtain clearer ultrasonography imaging after switching from gel to alcohol.

While the results of this study offer a potential approach to early pregnancy detection and EBP validation in the anoa, we readily acknowledge the limitations due to the small sample size of only four animals. As such, we believe these data are more appropriately represented as a case study. Clearly, further replication is needed before these findings can be confirmed as a reliable approach for early pregnancy detection. Understandably, obtaining a larger sample size of the endangered anoa presents a real challenge, given the sparsity of available adult female anoas in captive settings. We hope to pursue this effort in the future as additional animals become available. Nevertheless, the results of this study are encouraging and suggest the potential value of transabdominal ultrasonography in combination with urine biochemical profiles as a minimally invasive technique for early pregnancy detection in anoas. In conclusion, this study suggests that biochemical profiles of urine and transabdominal ultrasonography offer a novel, minimally-invasive procedure for early pregnancy detection and EBP validation in anoas.

ACKNOWLEDGEMENTS

This research was funded by Faculty of Mathematics and Natural Sciences, Universitas Indonesia, through Hibah Riset FMIPA UI Tahun 2021 Grant (NKB 006/UN2.F3/HKP.05.00/2021). RCK's effort was supported in part by the National Institutes of Health (NIH) Office of Research Infrastructure Programs (ORIP) under award number P51OD010425 to the Washington National Primate Research Center, USA.

REFERENCES

Acosta TJ, Hayashi KG, Matsui M, Miyamoto A. 2005. Changes in follicular vascularity during the first follicular wave in lactating cows. J Reprod Dev 51 (2): 273-280. DOI: 10.1262/jrd.16092.

Ali A, Hayder M. 2007. Ultrasonographic assessment of embryonic, fetal and placental development in ossimi sheep. Small Rumin Res 73: 277-282. DOI: 10.1016/j.smallrumres.2007.01.011.

Alto WA. 2005. No need for glycosuria/proteinuria screen in pregnant women. J Fam Pract 54: 978-983.

Burton JA, Hedges S, Mustari AH. 2005. The taxonomic status, distribution and conservation of the lowland anoa *Bubalus depressicornis* and mountain anoa *Bubalus quarlesi*. Mammal Rev 35 (1): 25-50. DOI: 10.1111/j.1365-2907.2005.00048.x.

Burton J, Wheeler P, Mustari A. 2016. *Bubalus depressicornis*. The IUCN Red List of Threatened Species 2016: e.T3126A46364222.

Dejneka GJ, Ochota M, Bielas W, Niżański W. 2020. Dystocia after unwanted mating as one of the risk factors in non-spayed bitches-a retrospective study. Animals 10 (9): 1697. DOI: 10.3390/ani10091697.

Dijkhuizen TJ, van Eerdenburg FJ. 1997. Behavioural signs of oestrus during pregnancy in lactating dairy cows. Vet Q 19 (4): 194-196. DOI: 10.1080/01652176.1997.9694771.

Hohnholz T, Volkmann N, Gillandt K, Waßmuth R, Kemper N. 2019. Risk factor for dystocia and perinatal mortality in extensively kept angus suckler cows in Germany. Agriculture 9 (4): 85. DOI: 10.3390/agriculture9040085.

Ingawale MV, Bakshi SA, Birade HS, Chinchkar SR, Gulavane SU. 2012. Early detection of pregnancy using ultrasonography in buffaloes. Buffalo Bull 31: 202-208.

Johnson CA. 2008. Glucose homeostasis during canine pregnancy: Insulin resistance, ketosis, and hypoglycemia. Theriogenology 70 (9): 1418-1423. DOI: 10.1016/j.theriogenology.2008.09.008.

Jones AK, Gately RE, McFadden KK, Hoffman ML, Pillai SM, Zinn SA, Govoni KE, Reed SA. 2017. Ultrasound during mid-gestation: Agreement with physical foetal and placental measurements and use in predicting gestational age in sheep. Reprod Domest Anim 52 (4): 649-654. DOI: 10.1111/rda.12961.

Katiyar R, Prasad S, Gupta HP. 2015. Total protein and glucose profil of urine during early pregnancy in buffaloes (*Bubalus bubalis*). Life Sci Bull 12 (1): 73-75.

Khan MM, Prasad S, Khan FA, Gupta HP. 2015. Investigation of differences in biochemical and mineral composition of urine between pregnant and non-pregnant crossbred dairy cattle. Iran J Appl Anim Sci 5 (4): 787-792.

Kim MJ. 2015. Transrectal ultrasonography of anorectal diseases: Advantages and disadvantages. Ultrasonography 34 (1): 19-31. DOI: 10.14366/usg.14051.

Kurzweil A, Martin J. 2020. StatPearls Publishing. https://www.ncbi.nlm.nih.gov/books/NBK534813/

Mannion CA, Gray-donald K, Koski KG. 2006. Association of l transabdominal ultrasound. ow intake of milk and vitamin D during pregnancy with decreased birth weight. Can Med Assoc 174 (9): 1273-1277. DOI: 10.1503/cmaj.1041388.

Mayasari A, Suryawan A, Christita M, Simamora ATJ, Abinawanto A, Suryanda A, Bowolaksono A. 2018. Vaginal swab cytology aplication to determine the estrous cycle of lowland anoa (*Bubalus depressicornis*, smith, 1927) in captivity. MATEC Web Conf 197: 06008. DOI: 10.1051/matecconf/201819706008.

Medan MS, Abd El-Aty AM. 2010. Advances in ultrasonography and its applications in domestic ruminants and other farm animals reproduction. J Adv Res 1 (2): 123-128. DOI: 10.1016/j.jare.2010.03.003.

Miranda SA, Leão DL, Oliveira KG, Sodré IS, Domingues SFS. 2018. Gestational ultrasonography and dopplerfluxometry in capuchin monkeys (*Sapajus apella*) zoometric. Theriogenology 108: 63-73. DOI: 10.1016/j.theriogenology.2017.11.023.

Naikoo M, Patel DM, Derashri HJ. 2013. Early pregnancy diagnosis by transrectal ultrasonography in mehsana buffaloes (*Bubalus bubalis*). Buffalo Bull 32 (2): 120-125. DOI: 10.14456/ku-bufbu.2013.19.

Persson B, Hansson U. 1993. Hypoglycaemia in pregnancy. Baillieres Clin Endocrinol Metab 7 (3): 731-739. DOI: 10.1016/s0950-351x(05)80216-2.

Petrujkić BT, Cojkić A, Petrujkić K, Jeremić I, Mašulović D, Dimitrijević V, Savić M, Pešić M, Beier RC. 2016. Transabdominal and transrectal ultrasonography of fetuses in württemberg ewes: Correlation with gestational age. Anim Sci J 87 (2): 197-201. DOI: 10.1111/asj.12421.

Rawat P, Bathla S, Baithalu R, Yadav ML, Kumar S, Ali SA, Tiwari A, Lotfan M, Naru J, Jena M, Behere P, Balhara AK, Vashisth R, Singh I, Dang A, Kaushik JK, Mohanty TK, Mohanty AK. 2016. Identification of potential protein biomarkers for early detection of pregnancy in cow urine using 2D DIGE and label free quantitation. Clin Proteom 13: 15. DOI: 10.1186/s12014-016-9116-y.

Romano JE, Christians CJ. 2008. Early pregnancy diagnosis by transrectal ultrasonography in ewes. Small Ruminant Res 77: 51-57. DOI: 10.1016/j.smallrumres.2008.02.004.

Schmid D, Schiess A, Tustain G, Fleisch A, Bollwein H, Janett F. 2016. Sonographic diagnosis of pregnancy and determination of fetal number and gestational age in ewes. Schweiz Arch Tierheilkd 158 (2): 93-101. DOI: 10.17236/sat00049.