International Journal of Agricultural Technology 2022Vol. 18(4):1753-1766
Available online http://www.ijat-aatsea.com
ISSN 2630-0192 (Online)

Determination of pregnancy associated - glycoproteins (PAGs) During and post pregnancy in riverine buffaloes (*Bubalus bubalis* Linn.)

Rigos L. M.[1*], Atabay, E. C. [2,3], Atabay, E. P [3] and Apolinario, J. P. R. [3]

[1]College of Veterinary Science and Medicine, Central Luzon State University; [2]Philippine Carabao Center at CLSU; [3]Reproduction and Physiology Section, Philippine Carabao Center, National Headquarters and Gene Pool, Science City of Munoz, Nueva Ecija, 3120, Philippines.

Rigos L. M., Atabay, E. C., Atabay, E. P and Apolinario, J. P. R. (2022). Determination of pregnancy-associated - glycoproteins (PAGs) during and post-pregnancy in riverine buffaloes (*Bubalus bubalis* Linn.). International Journal of Agricultural Technology 18(4):1753-1766.

Abstract Through pregnancy-associated glycoprotein assay, an overall conception rate of 54. 17% at days 25 and 30 post fixed-time artificial insemination (FTAI) was observed in the study. The pregnancy of the riverine buffaloes (n=13) was confirmed through transrectal ultrasonography (TRUS) at day 40, showing the presence of an amniotic vesicle and an embryo with a beating heart. The mean plasma level of pregnancy-associated glycoproteins (PAGs) of the pregnant buffaloes (n=11) were found at a high level as early as days 25, 30, and 40 (1.21 ± 0.20 ng/ml, 12.11 ± 1.67, and 28.81 ± 2.57 ng/ml) and observed to have increasing trend/pattern with the progression of pregnancy until day 300 (114.01 ± 10.05). Two of the animals that were confirmed pregnant at day 40 post artificial insemination (AI) via TRUS undergone early pregnancy loss. While the non-pregnant buffalo's plasma PAGs remained at a very low level on day 25 (0.17 ± 0. 04 ng/ml) to day 40 (0.06 ± 0.02 ng/ml) and further confirmed the non-pregnancy via TRUS. Two waves of peak increase in the concentration of PAGs were found during the gestation period of the riverine buffaloes. The 1st peak was observed during the 1st trimester (day 60) and the 2nd peak was during the last trimester (day 270) of gestation. Generally, the findings on PAGs at early and throughout the gestation period of riverine buffaloes appeared higher than those reported in other studies. Postpartum residual clearance of PAGs concentration in the maternal circulation of the buffaloes was found to be slowly decreasing from 114.33 ± 13.75ng/ml at week 1 to nadir at week 10 with a plasma level of 0.11 ± 0.01 ng/ml.

Keywords: Binucleate cells, Buffalo, Pregnancy diagnosis, Syncytiotrophoblast

Introduction

For Asian countries, the importance of buffaloes as livestock animals can not be understated because of their efficiency in converting fibrous undigestable foodstuffs into milk and meat which richly abound the countryside. Milk and meat are essential macromolecules in the diet and gustatory satisfaction of people. The increasing demand in the

* **Corresponding Author:** Rigos, L. M.; **Email:** lmrigos@clsu.edu.ph

populace's diet for dairy products flooded the market for imported milk and milk by-products as local sources cannot supply the demand. To ease such importation, the government made a resolute step in upgrading the Philippine carabaos (swamp buffaloes) through crossbreeding with the riverine type of buffaloes.

The riverine buffaloes are hailed "milk of fortune" for their bounteous milk production which best fit them in upgrading the traditionally called "beasts of burden" Philippine carabaos. The hybrid Philippine carabaos inevitably improved their milk production while retaining their draft attributes and are now called "beasts of fortune" as they still till, plow, and harrow the farmlands.

The reproductive performance of the buffaloes is affected by several factors like poor estrous expression, seasonal unproductiveness, late sexual maturity, low conception, and low calf production (Michelizzi *et al.*, 2010), resulting in a long calving interval that limits the full reproductive potential of this animal species. To resolve the occurrence of silent heat in buffaloes, synchronization of ovulation through Fixed-Time Artificial Insemination (FTAI) was introduced in the Philippines (Atabay *et al.*, 2017) and generally resulted in increased artificial insemination (AI) efficiency and conception in these animal species.

Another major contributory factor to the reproductive inefficiency in both the dairy cattle and buffalo is pregnancy loss; hence, early and accurate diagnosis of pregnancy is very vital in their breeding program. Pregnancy diagnosis tools must be fast, suitable, and low-cost (Karen *et al*., 2015) to ensure the early reintroduction of non-pregnant animals into the breeding cycle. Several methods are used in the diagnosis of pregnancy. Transrectal palpation (TRP) is the commonly and widely used means of detecting pregnancy and the age of the fetus. However, this method is only accurate from day 45 of pregnancy in buffaloes (Karen *et al*., 2007). Additionally, palpation of the undulations in the endometrium, amniotic vesicle determination, and slithering of the chorioallantoic membranes may increase the incidence of fetal mortality (Rao *et al*., 2013). Another method is transrectal ultrasonography (TRUS) which accurately diagnoses early pregnancy from day 29 post-breeding. It can also determine the twinning and even sex of the fetus. However, the instrument is expensive, requires the technical skill of the person reading the scanned image, and requires careful handling and maintenance. Similarly, it is invasive, the 1% embryonic loss is associated with this method. (Rao *et al*., 2013; Lucy *et al*., 2011; Karen *et al*., 2007).

The newest method of pregnancy detection is the examination of blood plasma for pregnancy-specific protein. The use of pregnancy-associated glycoproteins (PAGs) has been in existence for several years but has not been applied to a large extent to the present time. Preliminary studies indicate that PAGs can be effective in detecting pregnancy as early

as day 24 through blood (Reese *et al.,* 2017) because it is a specific embryo marker that can detect the presence or absence of a viable embryo in the uterus. PAGs are potent pregnancy indicators in domestic cattle and buffalo as they are expressed temporally during the conception period (Jerome, 2012; Commun *et al*., 2016). They are released into the maternal blood upon nidation (20 days) where they can be assayed by different radioimmunoassay (RIA) and enzyme-linked immunoassay (ELISA) systems (de Sousa *et al*., 2003). Likewise, PAGs can be an ideal method in the early and accurate determination of pregnancy status in cows, and may also identify cows that underwent pregnancy loss or with a greater risk for loss at the time of diagnosis. This is of paramount importance because the incidence of pregnancy loss (embryonic mortality or abortion) from early diagnosis (days 25 to 30 after AI) to term is high in lactating dairy cows (Prvanovic *et al.,* 2009; Giordano *et al*., 2012). ELISA test is a proven and inexpensive means of quantitative confirmation of a particular substance in blood plasma. PAG-ELISA in present investigations proved to be a convenient and reliable method of detecting pregnancy in livestock animals that can shortly become routinary in the dairy industry (Friedrich *et al*., 2010). Also, the use of blood examinations eliminate the discrepancy in accuracy of pregnancy detection associated with the competencies of persons performing the ultrasound or palpation per rectum. Hence, the study aimed to determine the plasma concentration of pregnancy-associated glycoproteins (PAGs) as early as day 25 post fixed-time artificial insemination (FTAI); the profile of plasma pregnancy-associated glycoproteins (PAGs) concentration throughout the gestation period; and the postpartum concentration of residual plasma pregnancy-associated-glycoproteins (PAGs) using the BioPRYN® ELISA. The objectives of the study were to determine the plasma concentration of pregnancy associated-glycoproteins (PAGs) during and post pregnancy in riverine buffaloes (*Bubalus bubalis* Linn.). It specifically determined the PAGs concentration at day 25 post fixed time artificial insemination (FTAI), trend of PAGs concentration throughout the gestation period, and PAGs weekly concentration ten weeks post-partum.

Materials and methods

Selection of animals and conduct of fixed-time artificial insemination

Animals having a body condition score (BCS) of not less than three and with at least one ovary equal to or greater than two cm in length or width were selected and subjected to the Controlled Internal Drug Release (CIDR) Ovulation Synch Protocol of Fixed-Time Artificial Insemination (FTAI) (Figure 1). In brief, CIDR insertion, and gonadotrophin-releasing hormone injection (GnRH, Cystorelin, 100 µg i.m.) were done

simultaneously on day 1 to induce ovulation of the dominant follicle. Prostaglandin (PGF2α, 25 mg i.m) was administered and CIDR was removed on day 7 to prevent expression of estrus before induced luteolysis. Human chorionic gonadotrophin (hCG, 2 ml chorulon) administration was done on day 9 to induce ovulation of the new follicles formed after luteolysis and artificial insemination on day 10/11 (AM/PM) 14-16 h after hCG injection in FTAI protocol.

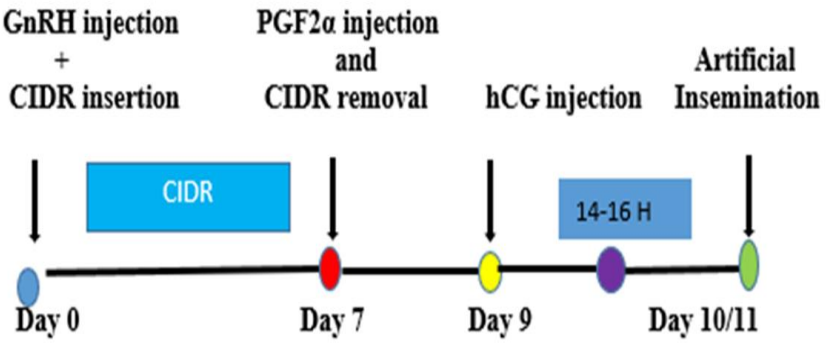

Figure 1. CIDR-Ovsynch Protocol of FTAI

Blood samples were collected from twenty-four riverine buffaloes that were subjected to FTAI at the Philippine Carabao Center (PCC), Central Luzon University, and PCC National Gene Pool. Assay of the plasma PAGs was done at the Hormone Assay Laboratory, Reproduction and Physiology Section, Philippine Carabao Center, National Headquarters and Gene Pool, Science City of Munoz, Nueva Ecija, Philippines. The study was conducted from February 2018 to May 2019.

Blood plasma samples

To detect early pregnancy, plasma samples were collected from the riverine buffaloes subjected to the CIDR-Ovsynch Protocol of FTAI at days 25, 30, and 40 post-FTAI. Blood was collected throughout the gestation period at days 60, 90, 120, 150, 180, 210, 240, 270, and 300, and from week 1 (day 1) until week 10 postpartum. Ten ml of blood on heparinized vacutainer tubes were collected via the jugular vein of the buffaloes.

Blood samples were centrifuged at 1,500 rpm for 20 minutes to separate the plasma and stored until assay. BioPRYN® ELISA kit (BioPRYN®, BioTracking) was used in the assay of PAGs.

Transrectal ultrasonography

All inseminated animals were observed for not returning to estrous and were subjected to pregnancy diagnosis via transrectal ultrasonography (TRUS) at day 40 post-FTAI to reconfirm pregnancy. Ultrasonographic

examination was performed using a transrectal ultrasound scanner equipped with a 7.5 MHz linear array transducer designed for intrarectal placement (Mehrajuddin *et al.,* 2013). The uterine horns were scanned on their dorsal and lateral surfaces. The positive pregnancy status of the animals was based on the presence of an amniotic vesicle and of an embryo (bean size = 2.0 to 2.5 cm) with a beating heart.

PAG/PSPB ELISA assay using BioPRYN® kit

PAG–ELISA is an antigen-capture or "sandwich" Enzyme-Linked Immunosorbent Assay (ELISA) that detects pregnancy-specific protein B (PSPB)/PAGs in the blood plasma. The assay was performed in wells of a polystyrene microtiter plate containing 96 (4 X 12) test wells coated with antiserum to PSPB/PAGs.

Before assay, plates, kit reagents, and plasma samples of the riverine buffaloes were warmed at room temperature and vortexed before use. A plate grid/map was prepared that corresponds to the 4 X 12 (= 96) wells plate. Animal identifications with their gestation period were assigned in each of the numbered wells in the grid to facilitate the reading and recording of the reading machine.

Plating was done by using a multichannel pipette in loading sample buffer # 1(50ml), PSB standards (W, X, Y, Z) control (150 ul) on the first column of the plates followed by plasma samples (150 ul) of the riverine buffaloes in all the wells of the plate, except for the column of the PSPB standards. Tips were changed each time the standards and plasma samples were loaded into the wells of the plate.

Plates were sealed with parafilm and were incubated for 1 hour at 37^0C. Following incubation, the contents of the plates were dumped and then blot dried in a paper towel. Plates were then set in an automatic plate washer and washed 4X in a 1X wash solution then blot dried again to remove any residual wash solution. With a multi-channel pipette, a quick detector (#2) and quick enhancer (#3) of 200 ul each were added to each well of the plates, sealed with parafilm, incubated for 30 minutes at 37^0 C every after the addition of the detector and enhancer and then washed 4X, then blot dried. TMB solution (#4) of 200 ul was then added to each well of the plates, covered with parafilm, and incubated for 15 minutes at 37^0C. Finally, a stop solution (#5) of 100 ul was added. This time, the contents of the plates were not dumped, washed, and blow-dried. Instead, they were mixed by swirling a few times before loading the plates in the plate reader machine and read at 650 nm. The strong color indicates binding and substrate reactivity of the labeled antibody conjugate to the bound PSPB/PAGs in the sample and is a positive indication of pregnancy. Weak color development indicates little or no binding of the labeled conjugate due to the absence or minimal amount of PAGs in the sample.

Readings were then exported as a text file in the Skanlt Software to generate and interpret the results. The value of 0.5 and above was interpreted as pregnant and the value below 0.5 as not pregnant.

Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). Levene's t-test (0.05%) was used to determine the level of significant difference in the PAGs concentration among pregnant and non-pregnant riverine buffaloes. Analysis of Variance (ANOVA) was computed to determine the level of significance of the increase or decrease of PAGs concentration throughout the gestation period. While Trend analysis was conducted to assess the pattern of increase or decrease in PAGs concentration with the progression of gestation and post partum.

Results

The findings of the study showed an overall conception rate of 54. 17% at days 25, 30, and 40 posts FTAI. Transrectal ultrasonography (TRUS) confirmed the pregnancy of the buffaloes (n=13) at day 40. Pregnant buffaloes revealed the presence of an amniotic vesicle and embryo with a beating heart. Conversely, two of the detected pregnant buffaloes by PAGs-ELISA assay on days 25 and 30 exhibited an absence of amniotic vesicle and an embryo without a beating heart at TRUS. The animals had an embryonic loss.

Results disclosed a significant difference ($p<0.05$) in the plasma circulating pregnancy-associated glycoproteins (PAGs) in the pregnant and non-pregnant riverine buffaloes. In pregnant buffaloes, the mean plasma PAG levels increased significantly from 1.21 ± 0.20 ng/ml on day 25 to 12.11 ± 1.67, and 28.81 ± 2.57 ng/ml on days 25, 30, and 40, respectively. Corollary, the mean PAG levels of the non-pregnant buffaloes remained very low on day 25 at 0.17 ± 0. 04 ng/ml, 0.13 ± 0.03 ng/ml, and 0.06 ± 0.02 on day 30 and 40, respectively. The gathered data of plasma PAGs assay showed the circulating PAGs concentration of pregnant and non-pregnant riverine buffaloes during early pregnancy period i.e., days 25, 30, and 40 post FTAI, respectively are presented in Table 1.

Table 1. Plasma PAG concentrations (ng/ml) of pregnant and non-pregnant riverine buffalo cows during early pregnancy (MEAN ± SEM)

Pregnancy status	**PAGs ng/ml Day 25**	**PAGs ng/ml Day 30**	**PAGs ng/ml Day 40**
Pregnant (P) (n=13)	1.21 ± 0.20*	12.11 ± 1.67*	28.81 ± 2.57*
Not pregnant (NP) (n=11)	0.17 ± 0. 04	0.13 ± 0.03	0.06 ± 0.02

* all means in pregnant buffaloes were significantly different at $p<0.05$ level of significance

Throughout the gestation period of the pregnant riverine buffaloes, their plasma PAGs concentrations revealed a significant ($p < 0.05$) increasing trend with the progression of pregnancy (Figure 2). Mean PAGs concentration significantly increased from 1. 21 ± 0.20 ng/ml, 12.11 ± 1.67 ng/ml, 28. 81 ± 2.57 ng/ml, 58.13 ± 10.67 ng/ml, 59.18 ± 9.55 ng/ml, 66.08 ± 9.64 ng/ml, 72. 69 ± 9.34 ng/ml, 82. 87 ± 7.12 ng/ml, 100. 62 ± 12.64 ng/ml, 113.63 ± 10.52 ng/ml, 128.66 ± 15.00 ng/ml, and 114.01 ± 10.05 ng/ml at days 25, 30, 40, 60, 90, 120, 150, 180, 210, 240, 270, and 300, respectively. It can be noticed that PAGs concentration decreased from day 270 towards day 300, post FTAI. Two waves of peak increase in the plasma concentration of PAGs were observed during the gestation period. The 1st peak was during the 1st trimester (day 60) and the 2nd peak was during the last trimester (day 270) of gestation. Trend analysis disclosed an increase in the circulating PAGs concentrations with time or progression of gestation.

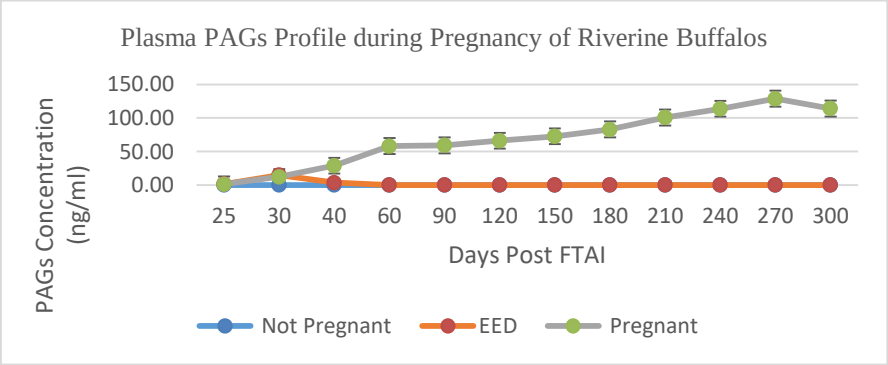

Figure 2. Trend analysis of plasma PAGs concentration throughout the gestation period of riverine buffaloes

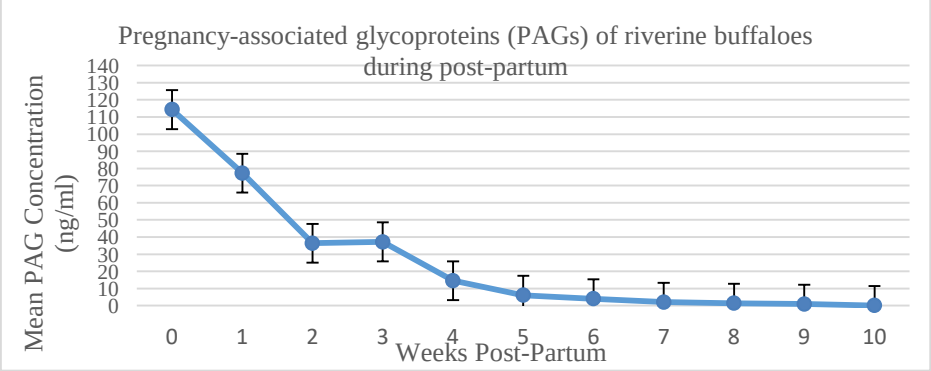

Figure 3. Trend analysis of residual plasma PAGs concentration post partum of riverine buffaloes

Findings on the pattern of clearance of residual PAGs in the maternal circulation of riverine buffaloes showed significant ($p < 0.05$) decreasing mean values of 114.33 ± 13.75, 77.26 ±10.36, 36.38 ± 5.15,

37.20 ± 10.25, 14.56 ± 2.76, 6.09 ± 1.69, 4.01 ± 1.15, 2.06 ± 0.29, 1.44 ± 0.21, 0.95 ± 0.16, and 0.11 ± 0.01 from week 0 (d1) to week 10, respectively (Figure 3). PAGs concentration slowly disappeared from week 1 to nadir at week 10. Trend analysis further confirmed that the reduction in PAGs concentration postpartum was logarithmic in a pattern.

Discussion

Pregnancy-associated glycoproteins (PAGs) are secreted by the mononucleate, binucleate, and multinucleated syncytial cells of the syncytiotrophoblasts of the placentome (Garbayo *et al*., 2000, Wallace *et al*., 2015) at the time of implantation at days 19-20 and throughout the gestation period (López-Gaitus *et al*., 2007; Balhara *et al*., 2013). The observed gradual significant increase in the PAGs level of the pregnant riverine buffalo cows from early pregnancy can be explained by the metamorphosis and proliferation of these PAGs secreting cells in the placentome. Placentome that markedly increased in number (Adeyinka, 2014) and size at the time of implantation and throughout the gestation period in buffalo (Ranjan and Singh, 2013; Schmidt *et al*., 2006).

The findings of the present study disagreed with the reported plasma PAGs concentration in cattle which increased slowly and remained at low levels during early pregnancy (Zoli *et al.,* 1992). Surprisingly, plasma PAGs concentrations in the pregnant riverine buffalo cows were higher than those observed in dairy cows from days 30 to 60 after breeding. A rapid increase in PAG concentrations associated with high maternal concentrations during early pregnancy is characteristic of caprine (Gonzalez *et al*., 2000) and ovine species (Ledezma-Torres *et al.,* 2006), and apparently of the riverine buffaloes.

The observed low level of plasma PAGs in the non-pregnant riverine buffalo cows on days 25, 30, and 40 conform with all other non-pregnant animals previously studied. This implied that PAGs can be a useful indicator for early and accurate determination of pregnancy. Accordingly, PAGs are not expressed throughout the gestation period, some are expressed early, while others appear later and are expressed over a shorter period of time (Barbato *et al.*, 2017). The PAGs family expressed very early in pregnancy (day 25) have a short half-life, these are shown in PAG-1, PAG-4, PAG-5, and PAG-9, making them ideal indicators for detecting early pregnancy (Commun *et al.,* 2016). Moreover, the high level of PAGs until day 40 was a manifestation of a successful pregnancy. Balhara *et al*. (2013) identified the embryonic period in cattle at about 42 days post insemination; hence, the pregnancy loss observed in the riverine buffalo cow at day 40 can be considered embryonic mortality (EM).

The increasing plasma PAGs concentration during the gestation period in riverine buffaloes can be related to the metamorphosis of the

mono-nucleate into binucleate cells (BNC) and multinucleated syncytial cells secreting PAGs during implantation in the endometrium (Wooding, 1983) and the enlargement of the placentome (Green *et al.,* 2005) brought about by the increase in size and weight, as stated in previous reports. Additionally, PAGs are also released from various tissues such as testicles, ovaries, fetal liver, salivary gland, colon, and granulocytes, hence the term "pregnancy associated-glycoproteins" (Barbato *et al*., 2017). Throughout the pregnancy period, the placentome number was markedly higher in the pregnant uteri than in the non-pregnant uteri at all stages of pregnancy (Schmidt *et al*., 2006), thus the high PAGs in the pregnant animals.

The occurrence of two waves of peak increase in the plasma PAGs concentration was also observed in the present study. The 1st wave of peak increase occurred during the 1st trimester of gestation which conforms with the observations in most cows (Wallace *et al*., 2015; Barbato *et al.,* 2017). However, a disagreement ensued on the 2nd wave of peak increase because, in the present study, the 2nd peak was at early of the last trimester of pregnancy, whereas Wallace *et al*. (2015) recorded a second peak at term in cows while Barbato *et al*. (2017) reported at around mid-trimester (d 240) post insemination in water buffaloes. The disparity can be related to breed specificity and, possibly, with the test kit since previous studies used RIA, while the present study used ELISA. However, this needs further evaluation and verification.

The observed declining plasma PAGs in riverine buffaloes from day 270 to day 300 disagrees with the findings of El-Battawy (2009) in Iraqui buffaloes wherein PAGs concentration on these animal species continuously increased towards parturition. However, observations of Shahin (2012) and de Sousa *et al*. (1999) in native goats corroborated the present findings, as PAGs on these small ruminants declined from week 19 (119 days) until parturition. The decline in the PAGs level might be related to the findings of Reynolds *et al.* (1990) in cows, wherein the weight of the placentomes regressed towards the end of pregnancy, tending to the decline in PAGs concentration.

Additionally, Shahin (2012) and de Sousa *et al.* (1999) reported a normal decrease in the BNC numbers before parturition which might have caused the reduction in the circulating PAGs in the riverine buffaloes. It can be further hypothesized that the observed reduction in PAG concentrations in pregnant riverine buffaloes was a result of both a species-specific pattern of PAGs secretion during their gestation, as well as from a better recognition of PAG epitopes by the BioPRYN® PAG-ELISA. It could also be that the decline at days 270 to 300 can be momentary as observed during the 1st peak of the plasma PAGs which maintained a steady level until day 90, before continuously increasing again. It might be that by day 300 to parturition, PAGs level may increase again; however, this needs further investigation.

Present findings recorded a higher concentration/level of PAGs than in other reported studies. Several studies related the number of fetuses to a positive effect on the PAGs concentration (Dobson *et al*., 1993). Twinning or multiple pregnancies, which are more frequent in sheep and goats, confirmed the effect of the increased PAGs concentration throughout pregnancy (Batalha *et al*., 2001; Vandaele *et al*., 2005; El Amiri *et al.*, 2015). Conversely, the normal singleton fetus in cows can be related to their lower PAGs concentration. Fetal gender has also been reported to influence the PAG levels in cows, ewes, and goats, where male fetuses have higher PAG levels than females (Zoli *et al*., 1992; Lopez-Gatius *et al.,* 2007). The present study neither agrees nor disagrees with the fetal gender effect on the PAGs concentration since this was not examined among the riverine buffaloes being studied.

As implicated in the results of plasma PAGs determination of present and previous studies, PAG is a useful test for monitoring pregnancy because any disturbance in the embryo/fetal status, i.e., embryo/fetal death, resulted in a disturbance in the placental function and the expression of placental products, such as PAGs. In case of fetal mortality, the concentration of PAG fell rapidly below the PAGs level of a normal pregnant animal of the same stage of pregnancy (Breukelman *et al*., 2005, Prvanovic *et. al.,* 2009). However, when maternal concentrations of PAGs have increased, the probability of maintaining pregnancy also increased (Pohler *et al.,* 2016).

PAGs, belong to the aspartic proteinase family that is released into the maternal circulation at the time of implantation, and that allow accurate pregnancy diagnosis. Likewise, since PAG molecules are products of the trophoblastic cells, their presence in the maternal blood is useful for predicting fetal well-being and is helpful in detecting early placental abnormalities, embryonic/fetal mortality, or abortion (López-Gatius *et al*., 2007).

Maintenance of pregnancy can probably be reflected on PAG concentrations and is influenced by both sire and sire breed used at FTAI. Variation in the incidence of pregnancy loss was detected among sires that could not be predicted with standard semen fertility evaluations. Exploring the relationship of sire and PAG production might be promising to improve sire selection concerning pregnancy loss (Franco *et al*., 2018). However, in the present study, sire and PAGs production concerning pregnancy maintenance were not evaluated.

Plasma PAGs concentration of riverine buffaloes throughout gestation exhibited individual differences among animals. One of the initially diagnosed pregnant buffalo cows with high circulating PAGs at days 25 and 30 showed embryonic loss as indicated by a marked decrease in the PAGs level and was confirmed by transrectal ultrasonography (TRUS) at day 40. The absence of an amniotic vesicle and a beating heart of the embryo was detected when the animals were subjected to confirmatory

TRUS. According to Lopez-Gatius *et al.* (2007), embryo/fetal loss can result in an abnormally high or low PAG concentration at day 35 of pregnancy, hence the detected embryonic death in the riverine buffalo.

Also, two riverine buffalo cows showed exceptionally high PAGs concentration but were not included in the general PAGs profile of riverine buffaloes. Similar aberrant observations were found in Zebu cows and taurine breeds and were also not included in their PAGs profile (de Sousa *et al*., 2003).

The observed embryonic loss in the riverine buffalo with high circulating plasma PAGs on days 25 to 30 but declined towards day 40 agrees with the study in beef cows (Pohler *et al*., 2016) that as PAGs concentration on day 31 decrease, there is a likelihood of undergoing embryonic mortality (EM) by day 59 of gestation. This is further supported by the observation of Thompson *et al*. (2010) in gestating dairy cows with greater plasma concentrations of PAG on day 30 after artificial insemination and underwent early embryonic mortality also (EEM) compared with cows undergoing late embryo mortality (LEM) between days 32 and 60 of pregnancy (4.98 ± 0.42 vs. 2.91 ± 0.96 ng/mL). Contrastingly, Franco *et al*. (2018) posited that dairy cows that underwent pregnancy loss have low PAGs concentration. This was corroborated by Pohler *et al*. (2013) whose study showed that an embryo loss after day 28 had reduced concentrations of PAG compared with cows that maintained pregnancy at day 28.

The findings on the slow clearance of PAGs concentration in the maternal blood of the riverine buffaloes studied from week 1 to week 10 can be related to the high concentration of PAGs on these animal species a day after parturition (114.33 ± 43.49). The relative slow clearance of residual PAGs was construed by the trend analysis which further implied that the reduction on PAGs concentration post partum was logarithmic in pattern.

Present and previous findings agree with the decreasing trend of PAGs concentration post partum, either by RIA and/or ELISA systems. However, one major problem of PAG-based pregnancy detection according to Zoli *et al.* (1992) is the long post partum serum half-life of PAGs. Protein glycosylation is an important factor that regulates plasma half-life. Variation in the plasma half-life of PAGs can be attributed to the variable degrees of glycosylation of these glycoproteins (Klisch *et al.*, 2005); hence, the variability of PAGs nadir in various animal species. Riverine buffalo PAGs reached a nadir in the 10^{th} week (70 days) post partum, water buffalo at 50 days post partum (Barbato *et al*., 2017), zebu cattle in the 10^{th} week (70 days) post partum (de Sousa *et al*., 2003), bovine in the 14^{th} week (100 days) post partum (Delahaut, 2017), and goat and sheep in the 4^{th} week (28 days) post partum (de Sousa *et al*., 1999).

Our present findings of 10 weeks or 70 days clearance of residual PAGs in the maternal blood of riverine buffaloes conform with the usual

waiting time of about 60 days dry period after parturition (Balhara *et al.*, 2013); hence, our findings can be used as a tool in detecting new pregnancy in riverine buffaloes.

In conclusion, pregnancy-associated glycoprotein (PAG) assay is a method of early and accurate detection of pregnancy. It detected pregnancy in riverine buffalo cows at day 25, through a single test of the blood plasma sample using the BioPRYN® ELISA kit. The pattern of plasma PAG concentrations that significantly increased with the progression of gestation is a useful indication of the fetoplacental status because rapid fall of the PAGs level beyond the detection of pregnancy is an indication of embryonic or fetal loss (abortion). This allows culling or early rebreeding and thereby increases calving rate corollary to high productivity of the animals and, consequently, to the increase in farmers' income. Moreover, detection of the pattern of the disappearance of residual PAGs following calving in the maternal circulation can be a useful tool in detecting new pregnancies in the riverine buffaloes. However, the specificity and sensitivity of PAG assay as a routine pregnancy diagnosis in buffaloes can be considered for further study and evaluation.

Acknowledgments

The authors would like to thank Central Luzon State University, Philippine Carabao Center, Department of Agriculture and Philippine Council for Agriculture, Aquatic and Natural Resources and Research Development, Department of Science and Technology for the funding of this study. Likewise, grateful recognition is due to Dr. Ramesh C. Tilwani, Dr. Jessica Gay M. Ortiz, Mr. Joselito V. del Rosario, and Mr. Rodante V. de Vera for their valuable help in the conduct of this study.

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(Received: 2 October 2021, accepted: 30 May 2022)