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Article

Using Blood Tests, Enzymes, and Hormonal Changes a.s Indicators to Determine the Sex of the Fetus in Ewes During Pregnancy

Fatima Juma Azgar[1], Qayssar J. Kadhim[2], Zaid H. Al-Zaidi*[3]

1. Surgery and Obstetric Department, College of Veterinary Medicine ,University of Kirkuk, Kirkuk, Iraq
2. Veterinary Department, Ministry of Agriculture, Iraq
3. Department of Forensic, College of Science, University of Kirkuk, Iraq

*Correspondence: zaid.hassan@uokirkuk.edu.iq

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Abstract: The ability to establish the fetal sex in sheep, as such an early time point, will not only serve as a significant administrative and economic tool for selecting future livestock production due to its applicability of rationalizing nutritional and grazing decisions based on male versus female gestation requirements; but moreover it provides great research significance because although the physiology between sexes during pregnancy is recognized yet remains largely misunderstood. The objective of this study was to determine whether fetal sex can be assessed in ewes by non-invasive monitoring the changes which occur during early, mid and late pregnancy on complete blood count (CBC), activity of liver enzymes and serum progesterone (P4) and oestrogen concentrations. Following synchronization of estrus by the use of vaginal sponges (Esponjavet) containing 60 mg medroxyprogesterone acetate, a study was carried out on fifteen healthy Iraqi ewes aged between two and four years with an average weight around 45 kg. Five consecutive blood samples were also taken over the course of gestation, as well as a control sample: embryo sex was diagnosed by ultrasound. Of these results were that of 6 ewes had males and 9 females. Significant differences ($p<0.05$) between male and female groups were observed for various blood parameters such as indicators of WBC, LYM%, GRA%, MCV, MCH, RDW,PLT and PCT& concentrations of iron and calcium respectively. Also, progesterone and estrogen concentrations as well as the activity of AST, ALT, and ALP enzymes were significantly different depending on pregnancy stage along with fetal sex. It should be noted that other well known diagnostic methods like laparotomy and laparoscopy, may identify these non-surgical ways of determining fetal sex but require larger sample size to validate reliability of the indicators & suggest utilization as proper spectral tools over conventional means in ewes.

Keywords: Ewes, Fetal Sex, Blood Profile, Progesterone, Estrogen, Liver Enzymes, Pregnancy

1. Introduction

Cow and sheep rearing plays a significant part of the monetary way to go for Iraq, as it is crucial food source further dairy animal items' meat wool with regards to what many ranchers rely on in rustic regions alongside semi dried conditions. Achievement of high sheep production programs are depended more on precise reproductive management and among its most significant factors is early pregnancy diagnosis and gender determination of fetus. This will enable the breeder to fine-tune his/her nutritional or

grazing program for either male- (ram) or female-carrying offspring, and may lead to a decrease in perinatal mortality as well as increased flock productivity [1-3]. At present, ultrasound imaging is the most widely used diagnostic technique for pregnancy diagnosis and fetal sexing in sheep [4,5]. However, its accuracy depends on a number of factors such as the term of gestation; fetal position (cephalic vs breech) and that means experienced operator and equipment availability. This has led researchers to look for more additional physiological and biochemical markers that can be used in conjunction with or as substitutes of radiographic examination under field conditions scarce resources [4-6].

More recently, studies on other non-human ruminants have shown large differences in a variety of hormonal and enzyme markers found in the mother's blood between carrying males rather than females. The mean testosterone concentrations and alkaline phosphatase (ALP) enzyme activity were significantly greater in female than male fetuses, but these indicators exhibited a strong positive correlation as to whether the camel would give birth with males [6]. Similar calf sex indicators have also been used to gain similar results in cows, where the hormones of male foetuses were measured within maternal plasma [7,8].

Progesterone plays one of the most important role in maintenance of pregnancy in ewes as steroid hormone [9–11]. Initially secreted by the corpus luteum, the placenta contributes a substantial portion of progesterone secretion starting around day 50 of pregnancy [10-12]. Conversely, estrogen levels increase gradually throughout gestation as the functional activity of the embryonic-placental unit rises [10-13]. In ewes, recent studies have implicated the concentrations of progesterone as related to both number and state (single or twin) [11-14]. Still, the association of these hormones with the specific sex such as female or male in sheep is less explored; this requires further research.

In contrast, the complete blood count undergoes major physiological variations during pregnancy in ewes including a persistent reduction of hemoglobin concentration and packed cell volume at mid-pregnancy caused by hypervolemia (physiological hemodilution), gradual increment of white blood cell differential as gestation progresses, rowing changes in platelet indices also related to acute inflammatory state induced by labor [15-17]. The maternal liver is also under increased metabolic load to viably support the growth demands of both a normal healthy viable pregnancy and fetus (Table 1) — some parameters including liver enzymes (AST, ALT and ALP) are known to be affected by it [18-19]. Furthermore, the effects of this metabolic response variation with sex-specific fetal gender has not been adequately studied during pregnancy in a breed specific manner such as Iraqi local ewes [15-20].

Therefore, the aim of this study was to assess shifts in concurrent complete blood count with iron & calcium levels and liver enzymes (AST, ALT, ALP), as well as serum progesterone and estrogen concentrations over five successive periods during gestation in single pregnant ewes versus a respective control sample while investigating these indicators between male-embryo-carrying compared female- embryo-carrying sheep at all stages toward exploring the utility of using these hematological/biochemical estimators alone or jointly for potential non-invasive supportive indicate fetal gender live detection by complementing through currently available anamnestic diagnostics can helping decision making management practices

2. Materials and Methods

Animals and Experimental Design

In this study, (15) clinically healthy local ewes, free from reproductive diseases, were used, with ages ranging between two and four years and an average weight of about (45) kilograms. The experiment began on 14/6/2023, and the last blood sample was taken on 23/11/2023. Estrus synchronization among the ewes was achieved using vaginal sponges (Esponjavet, Iranian-made) loaded with 60 mg of medroxyprogesterone acetate (MAP),

which were inserted for 12-14 days according to the approved protocols for estrus synchronization in sheep [21-22].The sponges were then removed, followed by natural mating. The sex of the embryos was determined and the pregnancy status was confirmed using the veterinary ultrasound device (SonoSite VET 180 Plus) through abdominal examination.

Blood Sample Collection

Blood samples were drawn from the jugular vein of each ewe in five consecutive stages distributed over the gestation period (from the first draw to the fifth draw), in addition to a control sample (pre-pregnancy/baseline). Each sample was divided into two parts: one part was preserved in tubes containing an anticoagulant (EDTA) for a complete blood count test, and the other part was left to clot and then the serum was separated by centrifugation to measure minerals, hormones, and enzymes. The serum was stored at a temperature of (-20°C) until the analyzes were conducted.

Laboratory tests and devices used

A complete blood count (WBC, LYM%, MON%, GRA%, RBC, HGB, HCT, MCV, MCH, MCHC, RDW-CV, RDW-SD, PLT, PCT) was performed using a high-precision automated hematology analyzer (Hematology Analyzer, GenoTEK Nano 3) with a testing speed of approximately 40 seconds per sample. The concentrations of iron (Fe) and calcium (Ca) were measured using a Chemistry Analyzer through the spectrophotometric method. As for the concentrations of progesterone and estrogen hormones, they were measured using the Electrochemiluminescence Immunoassay (ECLIA) technique with the Roche Cobas e411 device and the commercial Elecsys Progesterone III and Elecsys Estradiol III kits, which are highly sensitive and specific techniques widely used in veterinary and medical laboratories. The activity of liver enzymes GOT (AST), GPT (ALT), and ALP was measured using the kinetic colorimetric method with the same chemical analyzer.

Statistical Analysis

The ewes were divided based on the results of the ultrasound examination into two groups: a group carrying male embryos (n = 6) and a group carrying female embryos (n = 9). The mean and standard deviation (Mean ± SD) for each of the studied indicators were calculated within each of the five withdrawal phases and the control sample. The differences between the male and female groups were compared using the independent-samples t-test with Welch's correction for unequal variances, and a statistical significance level of ($p<0.05$) was adopted. The statistical analysis and the creation of the graphs were conducted using the Python programming language (SciPy and Matplotlib libraries).

3. Results

The total number of ewes used in the study was (15) ewes. Ultrasound examination showed that (6) of them (40.0%) were carrying male fetuses, while (9) ewes (60.0%) were carrying female fetuses (Figure 1). This ratio is close to the expected natural distribution of lamb genders in sheep, with a slight inclination toward females in this limited sample.

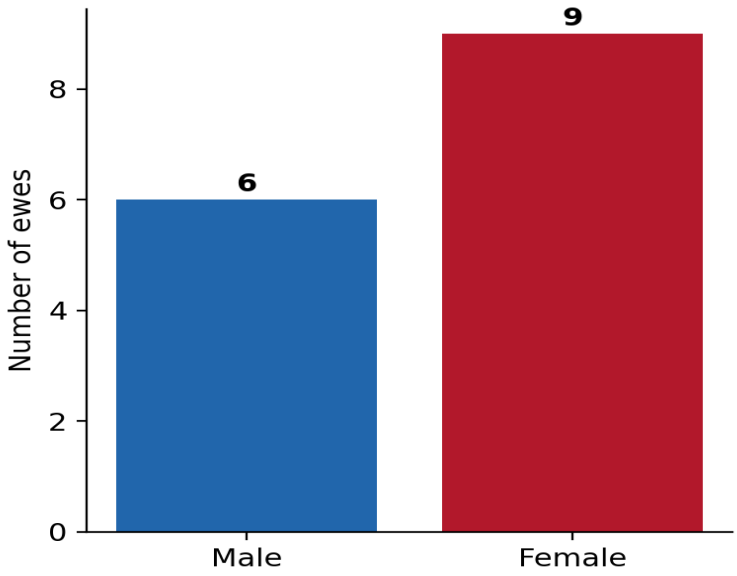


Figure 1. Distribution of fetal sex (male vs. female) among the studied ewes (n = 15).

The tables (1) below show the means ± standard deviations for all studied blood picture, mineral, hormone, and enzyme indicators, comparing ewes carrying male embryos and those carrying female embryos, at each of the five sampling stages and at control, with the p-value resulting from the t-test; cells with statistically significant differences ($p<0.05$) are shaded and marked with an asterisk (*).

Table 1. Comparison of hematological and biochemical indicators between ewes carrying male and female fetuses at the first draw (beginning of pregnancy)

Parameter	Male Fetuses (n=6) Mean ± SD	Female Fetuses (n=9) Mean ± SD	p-value
WBC (10^3/μL)	17.65 ± 4.09	23.34 ± 9.64	0.143
LYM%	88.48 ± 4.26	89.97 ± 1.75	0.449
MON%	7.48 ± 2.98	7.54 ± 2.80	0.969
GRA%	4.08 ± 1.37	3.30 ± 0.67	0.236
RBC (10^6/μL)	8.76 ± 0.08	8.76 ± 0.54	0.984
HGB (g/dL)	8.05 ± 0.27	7.80 ± 0.51	0.242
HCT%	24.43 ± 0.22	24.21 ± 2.09	0.760
MCV (μm³)	28.08 ± 0.39	27.60 ± 1.33	0.328
MCH (pg)	9.15 ± 0.27	8.89 ± 0.19	0.076
MCHC (g/dL)	33.13 ± 1.37	32.23 ± 0.89	0.194
RDW-CV%	17.78 ± 0.76	16.99 ± 0.38	0.051
RDW-SD (μm³)	18.22 ± 0.67	17.43 ± 0.89	0.076
PLT (10^3/μL)	878.50 ± 140.51	1158.11 ± 487.76	0.136
PCT%	1.02 ± 0.21	1.46 ± 0.67	0.095
Fe (μg/dL)	145.35 ± 16.31	124.25 ± 16.57	0.033*
Ca (mg/dL)	10.26 ± 0.56	10.63 ± 0.37	0.193
Progesterone (ng/mL)	1.27 ± 0.71	1.60 ± 1.89	0.652
Estrogen (pg/mL)	40.54 ± 20.31	58.21 ± 20.17	0.127
AST (U/L)	108.28 ± 3.60	113.98 ± 13.00	0.243
ALT (U/L)	29.05 ± 2.10	28.49 ± 3.93	0.726
ALP (U/L)	174.75 ± 57.82	97.22 ± 13.20	0.021*

Table 2. Comparison of the Studied Parameters between Ewes Carrying Male and Female Fetuses at the Second Blood Sampling

Parameter	Male Fetuses (n=6) Mean ± SD	Female Fetuses (n=9) Mean ± SD	p-value
WBC (10^3/μL)	26.95 ± 1.77	21.01 ± 2.75	0.000*
LYM%	92.98 ± 3.18	84.69 ± 6.93	0.009*
MON%	4.75 ± 2.10	7.61 ± 2.37	0.030*
GRA%	2.25 ± 1.13	7.73 ± 4.60	0.007*
RBC (10^6/μL)	8.26 ± 0.33	7.76 ± 1.92	0.462
HGB (g/dL)	8.32 ± 0.46	7.24 ± 2.15	0.182
HCT%	25.48 ± 0.35	22.40 ± 5.69	0.143
MCV (μm³)	30.92 ± 1.06	28.84 ± 0.99	0.003*
MCH (pg)	10.05 ± 0.20	9.50 ± 0.34	0.003*
MCHC (g/dL)	32.58 ± 1.52	31.52 ± 3.12	0.398
RDW-CV%	17.62 ± 0.44	16.46 ± 0.28	0.001*
RDW-SD (μm³)	21.22 ± 0.73	18.58 ± 0.60	0.000*
PLT (10^3/μL)	711.50 ± 111.79	599.67 ± 124.95	0.096
PCT%	0.69 ± 0.18	0.84 ± 0.28	0.227
Fe (μg/dL)	147.85 ± 23.48	120.94 ± 22.93	0.051
Ca (mg/dL)	10.54 ± 0.18	10.46 ± 0.40	0.612
Progesterone (ng/mL)	3.12 ± 0.34	3.62 ± 0.51	0.038*
Estrogen (pg/mL)	55.95 ± 15.81	63.55 ± 20.24	0.431
AST (U/L)	84.91 ± 12.54	88.73 ± 15.23	0.605
ALT (U/L)	22.20 ± 8.91	27.80 ± 4.73	0.201
ALP (U/L)	126.50 ± 29.78	177.22 ± 27.36	0.007*

Table 3. Comparison of the Studied Parameters between Ewes Carrying Male and Female Fetuses at the Third Blood Sampling (Mid-Gestation)

Parameter	Male Fetuses (n=6) Mean ± SD	Female Fetuses (n=9) Mean ± SD	p-value
WBC (10^3/μL)	30.12 ± 0.70	33.99 ± 5.30	0.061
LYM%	90.08 ± 2.73	93.23 ± 2.20	0.042*
MON%	6.23 ± 1.27	4.19 ± 0.62	0.009*
GRA%	3.72 ± 1.48	2.61 ± 1.72	0.209
RBC (10^6/μL)	9.24 ± 0.21	9.03 ± 0.28	0.109
HGB (g/dL)	9.22 ± 0.13	8.88 ± 0.23	0.003*
HCT%	28.00 ± 0.71	27.57 ± 0.62	0.250
MCV (μm³)	30.55 ± 0.69	30.67 ± 1.38	0.833
MCH (pg)	9.95 ± 0.42	9.86 ± 0.38	0.666
MCHC (g/dL)	32.92 ± 1.23	32.31 ± 0.61	0.300
RDW-CV%	16.78 ± 0.62	17.18 ± 0.38	0.206
RDW-SD (μm³)	18.68 ± 0.75	18.47 ± 1.47	0.714
PLT (10^3/μL)	480.83 ± 109.90	443.11 ± 223.84	0.672
PCT%	0.52 ± 0.10	0.54 ± 0.18	0.777
Fe (μg/dL)	133.25 ± 23.84	148.13 ± 13.25	0.206
Ca (mg/dL)	9.53 ± 0.68	8.95 ± 0.17	0.091
Progesterone (ng/mL)	7.34 ± 0.15	8.06 ± 2.17	0.351

Estrogen (pg/mL)	58.47 ± 12.77	39.26 ± 4.03	0.013*
AST (U/L)	84.77 ± 13.14	100.41 ± 13.60	0.048*
ALT (U/L)	17.38 ± 2.67	23.38 ± 1.84	0.001*
ALP (U/L)	141.00 ± 32.40	131.11 ± 47.26	0.639

Table 4. Comparison of the Studied Parameters between Ewes Carrying Male and Female Fetuses at the Fourth Blood Sampling

Parameter	Male Fetuses (n=6) Mean ± SD	Female Fetuses (n=9) Mean ± SD	p-value
WBC (10^3/μL)	11.34 ± 3.52	7.84 ± 2.82	0.072
LYM%	41.62 ± 1.38	52.09 ± 8.28	0.005*
MON%	15.18 ± 6.21	20.08 ± 5.65	0.152
GRA%	43.23 ± 4.85	27.87 ± 9.98	0.002*
RBC (10^6/μL)	6.11 ± 0.34	5.87 ± 1.84	0.713
HGB (g/dL)	8.72 ± 0.17	7.58 ± 2.64	0.232
HCT%	28.28 ± 1.80	27.47 ± 8.97	0.797
MCV (μm³)	46.35 ± 0.41	46.20 ± 2.07	0.837
MCH (pg)	14.25 ± 0.52	12.44 ± 1.47	0.006*
MCHC (g/dL)	30.78 ± 1.38	26.83 ± 2.18	0.001*
RDW-CV%	18.18 ± 0.50	19.10 ± 0.52	0.006*
RDW-SD (μm³)	24.20 ± 1.01	26.60 ± 1.17	0.001*
PLT (10^3/μL)	659.17 ± 83.31	297.33 ± 85.40	0.000*
PCT%	0.87 ± 0.12	0.37 ± 0.18	0.000*
Fe (μg/dL)	110.12 ± 22.00	164.16 ± 19.63	0.001*
Ca (mg/dL)	11.78 ± 2.38	9.97 ± 0.12	0.122
Progesterone (ng/mL)	9.72 ± 2.75	11.53 ± 3.49	0.284
Estrogen (pg/mL)	76.95 ± 15.26	66.52 ± 18.85	0.260
AST (U/L)	65.50 ± 1.77	76.97 ± 12.39	0.024*
ALT (U/L)	16.45 ± 2.38	22.64 ± 5.20	0.009*
ALP (U/L)	135.50 ± 24.57	135.44 ± 22.43	0.997

Table 5. Comparison of the Studied Parameters between Ewes Carrying Male and Female Fetuses at the Fifth Blood Sampling (Late Gestation)

Parameter	Male Fetuses (n=6) Mean ± SD	Female Fetuses (n=9) Mean ± SD	p-value
WBC (10^3/μL)	10.65 ± 0.33	10.35 ± 0.77	0.320
LYM%	32.18 ± 3.48	37.52 ± 4.82	0.027*
MON%	15.78 ± 8.60	6.33 ± 4.40	0.043*
GRA%	52.05 ± 5.67	56.21 ± 4.94	0.174
RBC (10^6/μL)	6.87 ± 1.18	6.63 ± 0.44	0.655
HGB (g/dL)	9.16 ± 0.09	8.56 ± 0.43	0.003*
HCT%	34.54 ± 0.50	31.02 ± 2.20	0.001*
MCV (μm³)	46.85 ± 0.10	46.70 ± 0.25	0.136
MCH (pg)	12.48 ± 0.18	13.25 ± 0.57	0.003*
MCHC (g/dL)	26.58 ± 0.35	28.33 ± 1.35	0.004*
RDW-CV%	18.88 ± 0.23	18.29 ± 0.41	0.004*
RDW-SD (μm³)	27.80 ± 1.21	25.42 ± 0.89	0.003*
PLT (10^3/μL)	370.50 ± 19.81	457.33 ± 79.93	0.012*

PCT%	0.37 ± 0.01	0.53 ± 0.15	0.013*
Fe (μg/dL)	154.55 ± 10.56	133.00 ± 11.76	0.003*
Ca (mg/dL)	9.81 ± 0.29	9.48 ± 0.22	0.044*
Progesterone (ng/mL)	1.94 ± 0.23	1.94 ± 0.57	0.986
Estrogen (pg/mL)	168.27 ± 25.97	70.80 ± 86.18	0.010*
AST (U/L)	73.15 ± 4.31	81.68 ± 9.46	0.036*
ALT (U/L)	15.55 ± 0.89	14.62 ± 2.98	0.401
ALP (U/L)	92.00 ± 9.25	99.36 ± 25.77	0.450

Table 6. Comparison of the Studied Parameters between Male and Female Groups in the Control Group (Baseline)

Parameter	Male (n=6) Mean ± SD	Female (n=9) Mean ± SD	p-value
WBC (10^3/μL)	8.42 ± 1.16	8.76 ± 1.34	0.621
LYM%	65.40 ± 4.95	50.69 ± 7.64	0.001*
MON%	6.02 ± 0.38	6.46 ± 2.15	0.557
GRA%	28.75 ± 4.77	42.86 ± 5.85	0.000*
RBC (10^6/μL)	7.08 ± 0.12	6.47 ± 0.58	0.013*
HGB (g/dL)	10.36 ± 0.47	9.33 ± 1.44	0.074
HCT%	33.10 ± 0.85	29.53 ± 3.61	0.019*
MCV (μm³)	49.76 ± 7.19	46.74 ± 0.63	0.351
MCH (pg)	14.37 ± 0.95	14.26 ± 1.09	0.838
MCHC (g/dL)	31.45 ± 2.16	30.46 ± 2.03	0.394
RDW-CV%	20.26 ± 0.61	20.85 ± 6.19	0.783
RDW-SD (μm³)	27.16 ± 0.24	25.19 ± 0.60	0.000*
PLT (10^3/μL)	1075.00 ± 367.70	879.78 ± 143.17	0.261
PCT%	1.38 ± 0.54	1.05 ± 0.22	0.207
Fe (μg/dL)	159.80 ± 13.58	135.52 ± 18.09	0.011*
Ca (mg/dL)	11.26 ± 0.01	10.41 ± 0.41	0.000*
Progesterone (ng/mL)	37.50 ± 22.85	10.84 ± 11.47	0.035*
Estrogen (pg/mL)	9.87 ± 3.87	31.61 ± 7.10	0.000*
AST (U/L)	99.16 ± 9.09	82.78 ± 5.68	0.005*
ALT (U/L)	25.66 ± 2.16	22.95 ± 3.85	0.106
ALP (U/L)	72.17 ± 9.20	89.00 ± 26.45	0.108

When tracking the concentration of progesterone through the five stages of pregnancy (Figure 2), it was observed that its concentrations in ewes carrying female embryos were relatively higher than those carrying male embryos at the second draw (3.62 ± 0.51 vs. 3.12 ± 0.34 ng/ml; p=0.038). Meanwhile, the concentration of progesterone significantly increased in the control sample of ewes that later carried male embryos (37.50 ± 22.85 vs. 10.84 ± 11.47 ng/ml in those carrying females; p=0.035).

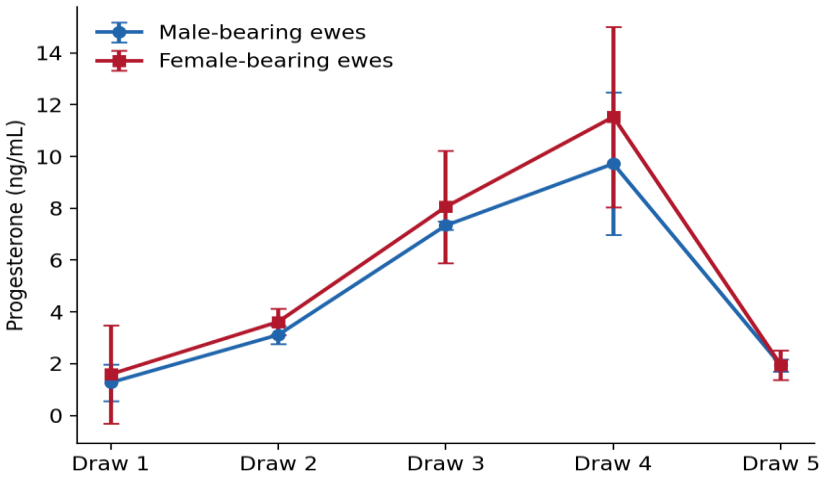


Figure 2. Serum progesterone concentration (mean ± SD) by fetal sex across the five sampling draws.

As for the concentration of estrogen (Figure 3), it showed a notable pattern in the late stages of pregnancy, as it significantly increased in ewes carrying male fetuses at the third sampling (58.47 ± 12.77 compared to 39.26 ± 4.03 picograms/ml in those carrying female fetuses; p=0.013) and continued this trend to the fifth sampling (168.27 ± 25.97 compared to 70.80 ± 86.18 picograms/ml; p=0.010). This pattern indicates relatively higher activity of the fetal-placental unit associated with the male fetus in the advanced stages of pregnancy in this sample.

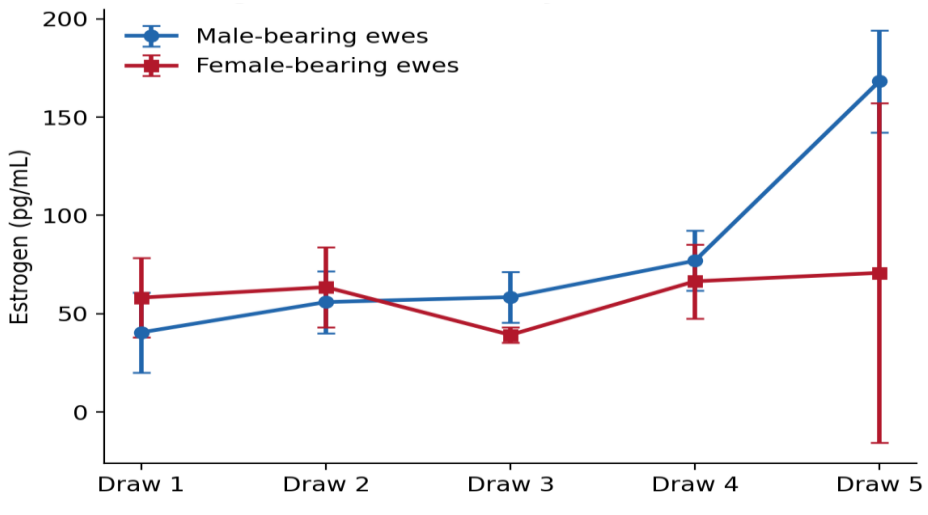


Figure 3. Serum estrogen concentration (mean ± SD) by fetal sex across the five sampling draws.

Regarding liver enzymes (Figures 4 and 5), significant differences were repeatedly observed between the two groups at the third, fourth, and fifth draws. The ALT enzyme activity was significantly higher in ewes carrying female fetuses compared to those carrying male fetuses at the third draw (23.38 ± 1.84 vs. 17.38 ± 2.67 units/liter; p=0.001) and the fourth draw (22.64 ± 5.20 vs. 16.45 ± 2.38 units/liter; p=0.009). A similar trend was nearly recorded in AST enzyme activity. A significant increase in ALP enzyme activity was observed in the first draw for ewes carrying male fetuses compared to those carrying female fetuses (174.75 ± 57.82 vs. 97.22 ± 13.20 units/liter; p=0.021). However, the trend reversed in the second draw (126.50 ± 29.78 for males vs. 177.22 ± 27.36 for females;

p=0.007), reflecting a phase-dependent dynamic in hepatic metabolic response according to the fetus's sex and the stage of pregnancy.

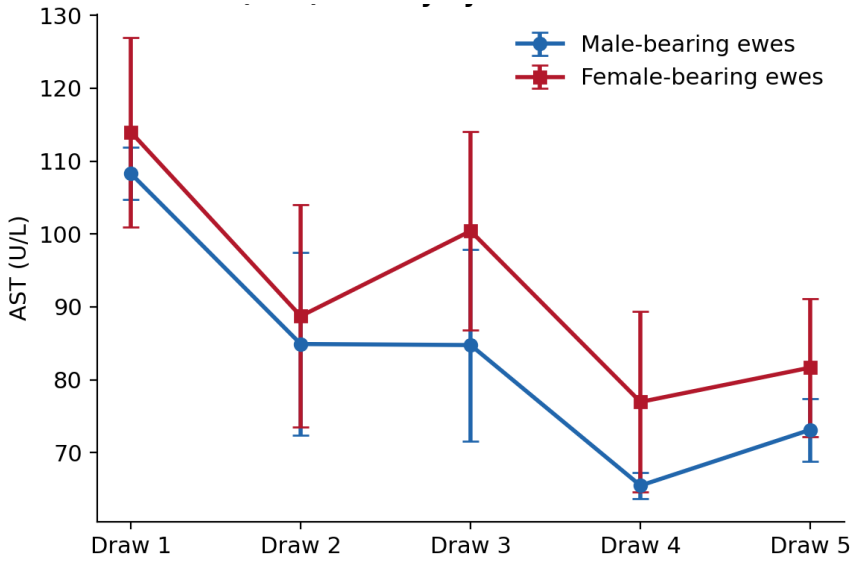


Figure 4. Serum AST (GOT) activity (mean ± SD) by fetal sex across the five sampling draws.

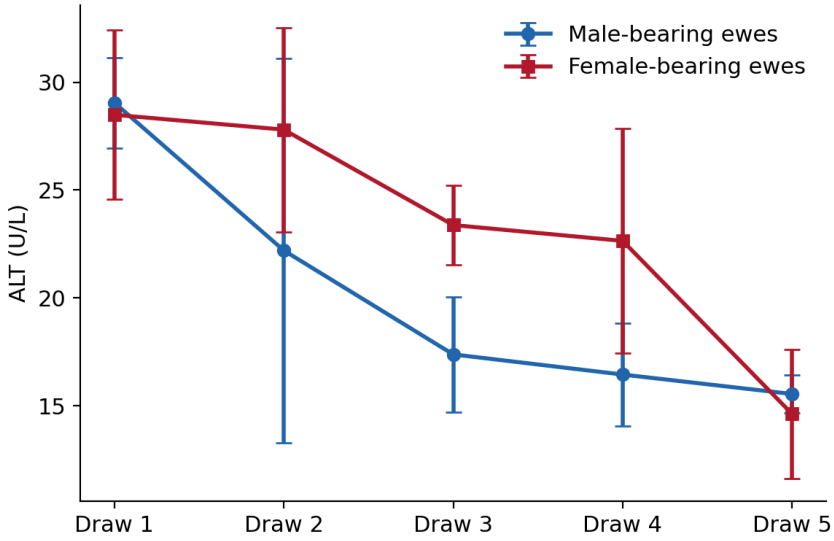


Figure 5. Serum ALT (GPT) activity (mean ± SD) by fetal sex across the five sampling draws.

In terms of the complete blood count (Figure 6), ewes carrying male fetuses showed a significant increase in total white blood cell count during the second draw (26.95 ± 1.77 versus 21.01 ± 2.75 $\times 10^3/\mu L$ for those carrying female fetuses; $p<0.001$). Meanwhile, platelet indices (PLT and PCT) showed significant differences during the fourth draw in favor of those carrying male fetuses (659.17 ± 83.31 versus 297.33 ± 85.40 $\times 10^3/\mu L$; $p<0.001$). The trend reversed during the fifth draw in favor of those carrying female fetuses (457.33 ± 79.93 versus 370.50 ± 19.81 $\times 10^3/\mu L$; $p=0.012$), reflecting a temporal variation in the maternal immune and coagulation response depending on the fetus's sex and the stage of pregnancy.

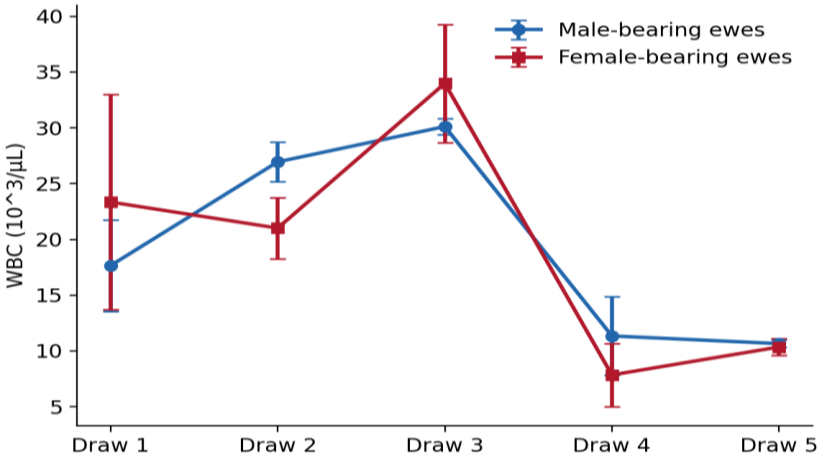


Figure 6. Total leukocyte count (WBC, mean ± SD) by fetal sex across the five sampling draws.

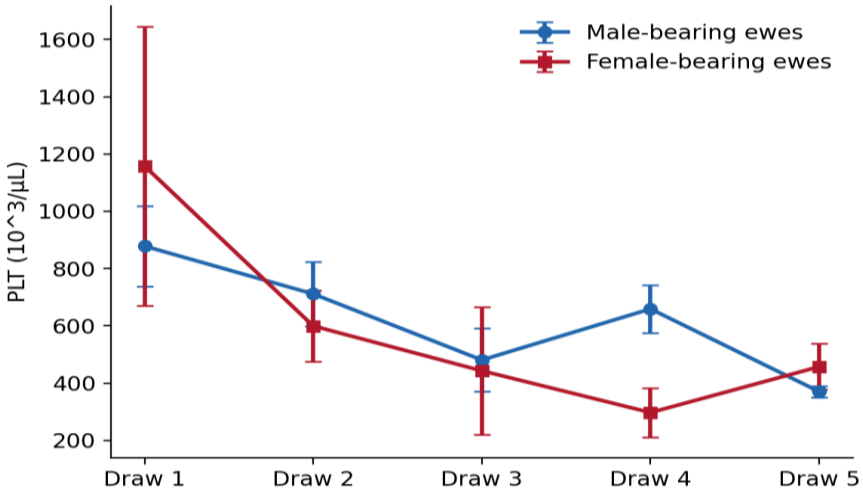


Figure 7. Platelet count (PLT, mean ± SD) by fetal sex across the five sampling draws.

4. Discussion

Using the reference for Aboelmaaty et al. (2024) pregnant camels study, our results are consistent with this general principlebased on protein and hormonal variation while carrying a male compared to holding female [1]. They reported that testosterone levels and activity of the ALP enzyme was significantly higher ($p<0.001$) in mothers with male foetuses, as well as a strong positive correlation between these two indices and male birthdate. By contrast, concentrations of cortisol, glucose and creatinine were significantly reduced in multiparous women carrying males than those carrying females [1]. This concurs with the current study where ewes that carried a male fetus had increased activity of this enzyme; however, its direction was reversed in later gestation stages potentially reflecting a species difference between camels and sheep in which sex-associated peak enzymatic activation occurred. As for progesterone, the serum concentration of this hormone in pregnant ewes increases from approximately Day 60 to Day 80 and remains elevated until just before parturition. This greater increase is more dependent on the number of fetuses and pregnancy milieu than on fetal sex; e.g Ozturan et al. Assaf ewes with analysis up to 2025 showed that the concentrations of progesterone in pregnant compared to non-pregnant ewe serum increased significantly from day twenty, and also correlated positively with number of fetuses (twins vs singles) [2]. This significant variation in our findings between different stages of withdrawal (higher progesterone

females second- but lower testosterone males control sample) is surprising and suggests that fetal sex may be a secondary determinant, such as number of fetuses combined with other firsthand determinants including gestational stage or seasonal condition. This requires increasing the sample size in order to effectively control for these overlapping variables with respect to sex. identified a significant positive association ($p<0.001$) between the number of lambs born and progesterone concentration measured at day 100 of pregnancy from studies involving 423 ewes over eight years with overlaps in single vs twin pregnancies being partially attributed to total weight of lamb(s)[3]. This is consistent with the hypothesis that progesterone represents a more sensitive measure of total embryo number or mass than sex per se, and should be considered when interpreting the large differences in mean values for progesterone between males and females as described here. At last, regarding estrogen, we have evidence for a clear and stable increase in carriers of males during the middle (third pull) to advanced stages of pregnancy (fifth pulls). This pattern is in accordance with the hypothesised differential steroid activity between males and females suggested by camel studies [1]. On a physiological basis, this may be due to differing aromatase activity in the embryonic-placental unit based on fetal genetic sex (XY vs XX). Reproductive physiology literature in ruminants supports a role for the early embryonic testis to have an impact on maternal hormonal environment through intermediary steroidal products that enter the maternal circulation by way of placental transfer [22].

For the complete blood count, results from our study differed in the direction of impacts on whiteblood cell and platelet counts based upon gestational age at which these measures were taken as wellas sex of offspring during pregnancy. This is in accordance with the report of Antunović et al. indicating that almost all red and white blood cell counts, platelet count as well as erythrocyte indices such as MCV; [mean corpuscular volume], MCH; [Mean cellular hemoglobin]; and MCHC were phase-dependent during pregnancy/lactation period in ewes reflecting relatively better maternal general physiological/physiologic state rather than solely fetal sex dependent effect on maternal haematological status per se.[4] A comprehensive review on this has been conducted by korelidou et al. mentioned that the blood profile of ewes is largely affected by factors such as age, stage of production and pregnancy status. At mid-pregnancy the lowest values of red blood cells, hemoglobin and haematocrit are usually noted due to physiology dilution from increased plasma volume [5]. This corresponds with the small relative reductions in HGB and HCT values observed at mid-sampling within certain groups of the present study.

Such observations also support the findings of Boushaki-Oudah et al., regarding Rembi ewes, which showed an important difference in hematological and biochemical parameters between pregnancy stages due to metabolic changes related with growth towards feto-placentjanocdomin plus [6]. Our current study is in line with the increase of AST and ALT activities across stages of pregnancy owing to increased metabolic burden on mother's liver as gestation progresses, where hepatic gluconeogenesis processes adaptively enhance themselves so that the biosynthetic demands for glucose by developing fetus are satisfied. The physiology of this process has been well characterized in the pregnant sheep since studies by Rudolf and colleagues examining the effects of cortisol on fetal hepatic gluconeogenesis.

The estrus synchronization protocol in this study (60 mg medroxyprogesterone acetate-loaded Esponjavet sponges) represents the same procedure that has been utilized recently to assess the efficiency of estrus synchronization on ewes under field conditions. This work revealed comparable pregnancy rates compared to protocols employing alternatives like CIDR and FGA, which corroborates the robustness of the experimental design employed for timing pregnancy over successive withdrawal phases in this study.

Though these results are promising, it is important to consider limitations such as the sample size of 15 ewes (6 males, 9 females) that impact generalizability and warrant caution when interpreting significant differences between groups especially in indicators with relatively high standard deviation values – for example estrogen concentration at fifth withdrawal within the female group. The omission of such important auxiliary variables (either single- or twin embryo) could have returned to some overlap in the interpretation of differences observed for any parameters recorded, aligned with what was evidenced on previous referenced studies that number embryos might be one main confounding factor occurring as progesterone should be heavily influenced [2,3]. Hence, it is advisable to follow-up studies with larger sample sizes controlled for the variable of number of embryos; and multivariable logistic regression analysis to assess mutually independent contributions from these indicators in blood, hormones & enzymes that were indicative on fetal sex.

5. Conclusion

The current study prefaced a variety of complete blood count measurements (WBC, LYM%, GRA%, MCV, MCH and RDW), platelet indicators (the number PLT and PCT); the concentrations iron[Fe]and Calcium; as well as hormone markers progesterone & estrogen level, activity liver enzymes AST among male and female embryos in ewes. The direction and size of these differences are significantly heterogeneous based on gestational age. The present results suggest that the monitoring of these hematological, biochemical and hormonal variables during successive periods (L1–3) and duration (P7; 0< PF <12 hrs), in concurrent with ultrasound imaging may represented as a practical non-invasive complementary tool for early detection of fetal gender in ewes. Nevertheless, it is suggested to conduct broader studies in the future with larger number of animals keeping constant factors such as numbers of embryos and seasonal variations until these results are confirmed along with obtaining most appropriate cut-off values for each one of these markers regarding male/ female discrimination.

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