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Volume 58 No. 1 January – July 2021

CONTENTS

Original Articles

ORIGINAL ARTICLE

ULTRASONOGRAPHIC FEATURES OF THE UTERUS AND OVARIES IN HOLSTEIN-SAHIWAL CROSSBRED DAIRY HEIFERS AT DIFFERENT PHASES OF THE ESTROUS CYCLE

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ABSTRACT

The ultrasound features of the uterus, ovaries, dominant follicles, and corpora lutea of crossbred dairy heifers were described in relation to the phases of the estrous cycle. Ten (10) Holstein-Sahiwal crossbred dairy heifers underwent a double injection synchronization program using Prostaglandin F2α, and sonograms of the uterus, ovaries, and its related structures (dominant follicles and corpora lutea) were obtained at each phase of the estrous cycle: estrus, metestrus, diestrus, and proestrus. B-mode sonograms were collected using an ultrasound machine equipped with a 7.5 MHz transrectal linear array scanner. Significantly higher measurements in the thickness of the left uterine horn were observed, revealing a thicker hyperechoic uterine wall during estrus in contrast to metestrus. The left uterine horn wall was significantly thicker during proestrus and thinnest during metestrus and diestrus. The ovaries appeared as a heterogeneously hypoechoic structure with the right ovary during proestrus having a significantly lower echo score, appearing less echogenic in contrast to other phases. The ovoid anechoic follicles at proestrus and estrus revealed lower echo scores than the hypoechoic corpus luteum at diestrus. The results of this study can serve as baseline information for further studies to improve reproductive performance in dairy heifers.

Keywords: *dairy heifers, estrous cycle, ovaries, ultrasonography, uterus*

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INTRODUCTION

One of the reproductive goals of a well-managed dairy farm is for the heifers to calve at 24 months of age. Hence, they should be bred for the first time when they reach 15 months of age with a bodyweight ranging from 250 to 300 kg, depending on the breed (PCARRD, 1981; Wathes *et al.,* 2014) and get pregnant. Reproductive traits and milk yield are factors that determine farm productivity and profitability as negative reproductive traits lead to significant economic losses because of failure to attain ideal production levels (Morrow, 1986; Kibar *et al.*, 2018). Cattle herds achieving a low age at first calving close to 23 to 25 months minimizes the non-productive period, leading to optimum economic performance, however, this practice is difficult to accomplish by most farms as it requires adequate growth and rigorous heifer management procedures (Wathes *et al.*, 2014). In the Philippines, the major constraints that hinder dairy cattle productivity include a shortage in dairy animals due to costly importation of live stocks, long calving intervals due to poor breeding management in reference to the lack of an accurate estrus detection protocol, repeat breeding, and poor ovarian function (Alejandrino *et al.,* 1999; Schiere, 2010). Assessment of the ovaries and the reproductive tract of heifers, particularly the uterus, is done by rectal palpation to determine if they have undergone puberty, and thus, ready for breeding. However, accurate functional changes in the uterus and ovaries during the estrous cycle, normal reproductive events, and reproductive pathologies are difficult to detect by rectal

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palpation due to inherent subjectivity of the technician (Taverne and Willemse, 1989; Hanzen *et al*., 2000; Quintela *et al*., 2012). Integrating the use of ultrasonography to assess the different conditions of the uterus and ovaries of heifers during the various phases of the estrous cycle will contribute to effective breeding management for dairy heifers.

Ultrasonography allowed researchers to conduct sound reproductive management decisions for the herd while maximizing its use for understanding certain reproductive events. The use of ultrasonography to monitor uterine and ovarian changes in cows and heifers during the estrous cycle in previous studies (Pierson and Ginther, 1988; Kastelic *et al.,* 1990; Ribadu and Nakao, 1999; Bonafos *et al.,* 1995; Chasombat *et al*., 2013) have not completely described the ultrasonographic features of the uterus and ovaries undergoing specific phases of the estrous cycle, with limited information obtained for crossbred heifers. These existing studies primarily focused on the ultrasonographic appearance of the uterus and ovaries at specific phases of the estrous cycle, namely the estrus and diestrus phases, in purebred cows and heifers. Continuous evaluation of the uterus and ovaries, two functionally dependent structures, throughout the entire estrous cycle will minimize structural variability as changes occurring during the metestrus and proestrus phases will also be included. Any differences between the ultrasonographic features and dimensions of the uterus and ovaries between purebred or crossbred heifers from earlier studies will be reported and will serve as a guide in evaluating the reproductive status and the optimum reproductive environment suited for breeding crossbred dairy heifers.

MATERIALS AND METHODS

Animals and synchronization protocol

Ten (10) apparently healthy crossbred dairy heifers (Holstein-Friesian x Sahiwal) 16 to 23 months of age, and weighing around 250 to 350 kg with good body condition scores (BCS 3/5) housed at Real Fresh Dairy Farm in Bay, Laguna, Philippines were used in this study. Holstein-Friesian x Sahiwal crossbreds account for 90 percent of the total herd population and were utilized mainly for convenience. The heifers were grazed rotationally in para grass pasture, with salt -mineral block supplementation and water available for consumption *ad libitum*. Fifteen kilograms of spent grain was given to each heifer per day in addition to the grazed pasture. Animals are vaccinated against Hemorrhagic Septicemia every six months and dewormed with Albendazole every three months given by drenching. Vitamins A, D, and E were injected intramuscularly to the animals monthly.

The protocol (Protocol No. 2019-0015) used in this study has been evaluated and approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Baños. Heifers were individually palpated to assess their reproductive status. The uterus and ovaries were palpated, and the presence of functional structures, namely the corpus luteum (CL) and follicles, on both the left and the right ovaries were also noted. All animals were found to have mature CL and underwent a double-injection estrus synchronization program using Cloprostenol (Vemedim Corporation, Can Tho City, Vietnam), a synthetic Prostaglandin F2α analog. Two (2) mL of the drug was given to each animal intramuscularly during the first day of the 15-day synchronization program. Eleven (11) days after the first injection, animals were given a second injection of the same hormone and heat detector patches (Bulls-i® Green Scratch Off Peel and Stick Heat Detector Patches, LIC®, Hamilton City, New Zealand) were applied on the rump. Seventy-two (72) hours after administration of the hormone, all animals were exhibiting signs of estrus upon observation of the changes in the color of heat detector patches and through vaginal speculum examination.

Ultrasound examination

Fecal material was manually removed from the rectum and rectal palpation of the reproductive tract in each heifer was performed. Ultrasound gel was applied on the transducer and an obstetrical glove was used to protect the transducer against damage. The ultrasound machine (Shenzhen Well. D Medical Electronics Co., Ltd. – Shenzhen City, China) was set to B-mode with a 7.5 MHz frequency. Heifers were examined on days 0 (estrus phase), 3 (metestrus phase), 11 (diestrus phase), and 18 (proestrus phase) of the estrous cycle, periods in which hormonal concentrations are at their peaks for the manifestation of the specific phase of the estrous cycle, and the maximum size of the CL and/or follicles embedded on the ovaries is attained in reference to existing literatures (Zemjanis, 1970; Kindahl *et al*., 1976; Morrow, 1986; Kastelic *et al*., 1990; Singh *et al*., 1997; Chasombat *et al*., 2013).

The cervix served as a landmark for

locating the uterine body due to the hyperechoic appearance of the plica circulares. The transducer was placed longitudinally on the dorsal surface of the uterine body before moving it laterally to cover the entire width of the body. The transducer was moved towards the dorsal surface of the horns near the bifurcation which was examined in oblique planes before retracting the uterus to palpate the ovary. The cranial pole and the hilus of the ovary were identified before placing the transducer on its dorsal surface. The distance between the transducer face and the ovary was minimized to prevent the disruption of signals and the formation of artifacts. Sonograms were printed using an analog video graphic ultrasound printer (Sony® UP-895 MD, Sony Corp., Tokyo, Japan) and scanned.

Assessment of uterine and ovarian dimensions and echogenicity

All measurements were obtained from the scanned sonograms using a graphic editor software program (Adobe Photoshop CS6, Adobe Systems, Inc., San Jose, CA, USA). Uterine diameter was measured using a calibrated ruler as the distance between the dorsal and ventral wall of the uterine body and horns. Uterine body and uterine horn thickness were measured as the near-end and far-end regions of the walls for both structures. For the ovaries, dominant follicle and CL, all measurements including thickness (cm), length (cm), area (cm^2), diameter (cm), perimeter (cm), and circularity of the ovaries were measured using a calibrated lasso tool. Values for circularity are approximation whether the structures indicate a perfect circle or not. A perfect circle has a value of 1.0.

Echogenicity of the uterine body, uterine horn, and ovaries and their associated structures were measured. Average echogenicity was obtained by taking into consideration the sizes of all structures before selecting a standardized rectangular region of interest per organ for computer-assisted image analysis. Histogram mean values for the echogenicity of the uterus, ovaries, and their associated structures were recorded and averaged using a graphic editor software program (Adobe Photoshop CS6, Adobe Systems, Inc., San Jose, CA, USA) by randomly selecting three areas from each structure. Echogenicity of the walls of the uterine body and horns were measured using a 2 x 2 mm and 1.5 x 1.5 mm region of interest, respectively, while echotexture scores of the lumen of the uterine body and horns were obtained following a 10 x 10 mm region of interest. A 5 x 5 mm region of interest was used to measure ovarian echogenicity while the CL and dominant follicle echotexture scores were obtained following a 3 x 3 mm region of interest.

Data analysis

The data for the dimensions and echo mean values of the uterus, ovaries, dominant follicle, and CL under the different phases of the estrous cycle was collected, and mean ± SD for the preceding parameters was determined. The homogeneity of variances between different measurements and echo mean values of the uterine and ovarian structures at different phases of the estrous cycle was analyzed using Levene's Test. Analysis of Variance (ANOVA) was conducted to check for differences between the collected means. Tukey's Honestly Significant Difference (HSD) test was also performed to determine which group means are significantly different if the F-value is proven to be statistically significant. To check for differences between the left and right ovaries, Paired Student T-test was done on all measurements obtained from both ovaries. All statistical measurements utilized a significant difference of $P<0.05$.

RESULTS AND DISCUSSION

Ultrasonographic features of the uterus

The diameter and thickness of the uterine body and horns were recorded (Table 1). No significant findings were observed in the wall thickness of the uterine body and right horn between the different phases of the estrous cycle. On the other hand, uterine wall thickness of the left horn was observed to have significant differences ($P<0.05$) between the different phases of the estrous cycle. Wall thickness during estrus in the left uterine horn agree with the findings of Pierson and Ginther (1988) in which enhanced wall thickness of the uterus indicates the occurrence of estrus in large ruminants. In comparison to the uterine wall during proestrus, wall thickness during metestrus is relatively thinner, supporting the findings by Pierson and Ginther (1988) wherein decreased wall thickness is expected one to three days post-ovulation as estrogen concentrations drop. Furthermore, wall thickness during diestrus supports the findings of Pierson and Ginther (1998) and DesCôteaux *et al.* (2010) wherein the absence of swelling and endometrial fluid causes the uterine wall to become thinner. Fluctuations in the circulating levels of progesterone and estrogen stimulate endometrial changes during proestrus, allowing the uterine wall to become thicker approximately three to four days before the onset of ovulation (Pierson and Ginther, 1987; Sugiura *et al*., 2018) similar to findings obtained in this study.

No significant changes in the diameter of the uterine body and horns during the entire estrous cycle in this study were observed, in contrast to studies wherein a smaller uterine diameter is expected during diestrus as a result of a less fluid-filled endometrium (DesCôteaux *et al*., 2010) in response to the release of progesterone; and a larger uterine diameter during estrus and before ovulation in response to an increase in intrauterine fluid (Pierson and Ginther, 1988). In contrast to cows with a gross uterine body diameter of 3 to 4 cm (Zemjanis, 1970), a smaller uterine body diameter was observed in this study. Values for the uterine body diameter is relatively greater compared to the obtained values for the left and right horn, similar to the gross observations of the uterine body and horns in non-pregnant slaughter cows by Arambulo (1991). Uterine horn diameter in this study is within the range established by Sorensen (1978) in purebred cows, revealing a gross approximation of the uterine horn diameter to be 0.75 to 1 inch or 1.905 to 2.540 cm. Moreover, a relatively smaller uterine diameter and a thinner uterine wall can be observed during estrus in crossbred dairy heifers, different from the findings by Acorda *et al.* (2015) in mature dairy water buffalos with more than one parity in estrus.

Differences in uterine dimensions may be due to the applied ultrasonographic technique and sensitivity of the animals to prostaglandin. Assessment of the changes in the measurements of the cranial, dorsal, and ventral diameters of the uterus can aid in the evaluation of uterine dimension, with the curved ventral section reported to be the thickest portion of the uterine horns throughout the entire cycle (Kähn and Volkmann, 2004) whereas ultrasonographic technique used for this study involved placing the transducer only on the dorsal surface of each uterine horn near the bifurcation. In addition, the location of intrauterine fluid in heifers may have also resulted in differences in uterine dimensions. The exact section of the uterus containing the greatest volume of intrauterine fluid during the estrous cycle remains to be unknown in heifers as of date. Furthermore, no significant relationship has been established between the ovary bearing the ovulatory follicle and/or CL on the amount of intrauterine luminal fluid secreted by the ipsilateral uterine horn in non-bred heifers (Kastelic *et al*., 1991).

Table 1. Ultrasonographic thickness and diameter (Mean±SD) of uterine body and horns in Holstein-Sahiwal crossbred dairy heifers at different phases of the estrous cycle.

Parameter	Estrous Cycle Phase			
	Estrus	Metestrus	Diestrus	Proestrus
Thickness (cm)				
Body	0.30±0.061	0.38±0.077	0.33±0.075	0.35±0.094
Left Horn	0.29±0.040[a]	0.23±0.048[b]	0.24±0.061[ab]	0.31±0.052[ac]
Right Horn	0.25±0.058	0.26±0.060	0.25±0.049	0.29±0.063
Diameter (cm)				
Body	3.20±0.660	2.91±0.356	2.73±0.302	2.82±0.364
Left Horn	2.42±0.257	2.40±0.274	2.24±0.214	2.23±0.159
Right Horn	2.45±0.319	2.30±0.195	2.38±0.285	2.29±0.344

Means with different superscripts among rows between periods of the estrous cycle are statistically different ($P<0.05$)

Same physiological and anatomical changes occur on the reproductive organs whether estrus is natural or synchronized with PGF2α, hence, measurements may not be affected (Wenkoff, 1986). Differences among the onset of peak estrus in individual crossbred heifers on Day 0 due to fluctuating estrogen levels possibly affected uterine diameter as measurements may have been obtained during early estrus. To induce a significant increase in intrauterine fluid, circulating estrogen must reach peak concentration level for visible changes in the uterine horn diameter to occur as observed by Sumiyoshi *et al.* (2014) in lactating Holstein-Friesian cows.

Echo mean values for the wall and lumen of the uterine body and horns showed no significant differences (Table 2). Throughout the entire cycle, the walls of the uterine body and horns appeared to be a hyperechoic band enclosing the heterogenous hypoechoic lumen with distinct anechoic areas (Fig. 1), coinciding with the ultrasonographic description of the ruminant uterus by Ribadu and Nakao (1999), DesCôteaux *et al.* (2010), and Acorda *et al.* (2015). Lumen echogenicity of the uterine body and horns showed no significant differences throughout the entire estrous cycle in contrast to findings by Pierson and Ginther (1998) and DesCôteaux *et al.* (2010) wherein transverse presentation of the uterus at diestrus showed a highly uniform echogenicity, different from the phases of proestrus, estrus, and metestrus. Ideally, the absence of endometrial fluid in the diestrus uterus is responsible for its homogenously grey echotexture. During per-estrus, the echogenicity of the uterus is reduced with scattered anechoic zones. Specifically, a more echogenic endometrium with greater anechoic regions as a result of uterine swelling and intrauterine fluid is expected during estrus (Pierson and Ginther, 1988; De Ramos *et al.*, 2010; DesCôteaux *et al.,* 2010).

Ultrasonographic features of the ovaries

No significant changes (P>0.05) were observed in the dimensions (thickness, length, area, diameter, perimeter, and circularity) of both ovaries throughout the different phases of the estrous cycle (Table 3). Diameter of the ovaries in crossbred dairy heifers is relatively larger in contrast to goats with an estimated ovarian

Table 2. Echo mean values (Mean±SD) of wall and lumen of uterine body and horns in Holstein-Sahiwal crossbred dairy heifers at different phases of the estrous cycle.

Parameter	Estrous Cycle Phase			
	Estrus	Metestrus	Diestrus	Proestrus
Uterine wall				
Body	167.60± 24.904	184.22± 19.669	190.31± 10.098	180.65± 15.201
Left horn	173.36± 18.794	172.63± 9.308	175.26± 8.664	174.42± 18.741
Right horn	188.18± 13.436	186.16± 11.827	186.19± 13.029	186.10± 10.006
Uterine lumen				
Body	95.07± 8.312	93.21± 16.153	84.52± 13.197	80.87± 12.684
Left horn	86.70± 12.530	82.47± 11.401	82.90± 13.146	75.13± 11.446
Right horn	87.96± 11.536	81.81± 11.898	78.06± 6.934	77.48± 10.789

No significant differences measured between periods of the estrous cycle (P>0.05).

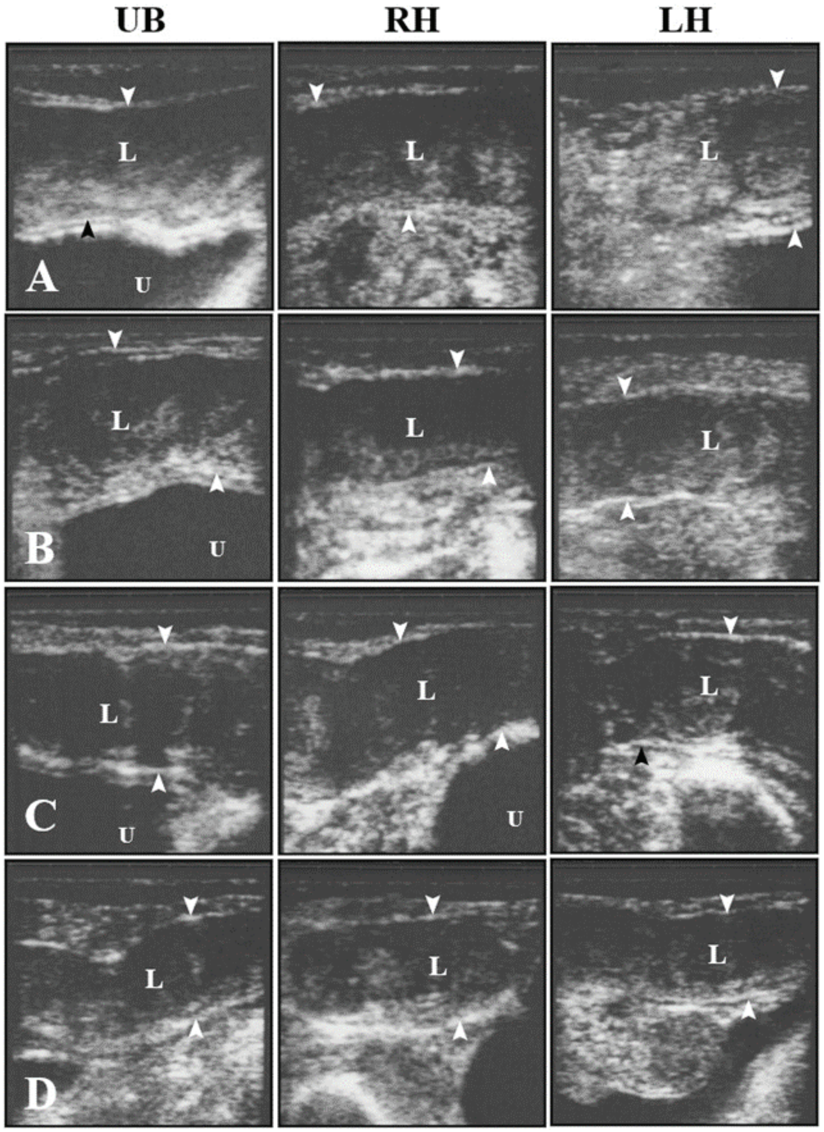

Figure 1. Representative longitudinal sonograms of the uterine body (UB), right uterine horn (RH), and left uterine horn (LH) of Holstein-Sahiwal crossbred dairy heifers at estrus (A), metestrus (B), diestrus (C), and proestrus (D). The uterine wall (arrowheads) appears as a thick hyperechoic band in all phases, with significant changes observed in the wall thickness of the LH. The urinary bladder (U) appears as an anechoic structure below the ventral wall of the uterine body.

diameter of 10 x 15 mm (Gonzalez-Bulnes *et al.*, 2010). To fully accommodate the number of differently sized follicles, ovary size increases concerning the occurrence of physiological changes during the estrous cycle (Acorda *et al.*, 2015).

Differences in ovarian length can be attributed to the smaller ovaries in buffalos, measuring at 3.0 cm x 1.4 cm x 1.0 cm in comparison to cows with ovaries estimated to measure at 3.7 cm x 2.3 cm x 1.5 cm; also, the minimal protrusion of the bubaline CL on the ovarian surface and differences in the CL diameter between buffalos (10 to 15 mm) and cows (12.5 to 25.0 mm) influences ovarian measurements (Drost, 2007). Antral follicle count (AFC) influences ovarian length and height as observed in crossbred beef heifers. High AFC heifers have greater ovary length measuring at 26.6 ± 0.05 mm as opposed to low AFC heifers with ovaries measuring 22.0 ± 0.13 mm while ovarian height for low and high AFC heifers was 12.4 ± 0.18 mm and 14.3 ± 0.04 mm, respectively (Cushman *et al.*, 2009).

Echo mean values for the left ovary were similar among all phases in contrast to the right ovary wherein echo mean values during proestrus were significantly lower ($P<0.05$) compared to other phases of the estrous cycle (Table 3). A hyperechoic border surrounding the ovarian

Table 3. Ultrasonographic dimensions and echo mean values (Mean±SD) of left and right ovaries in Holstein-Sahiwal crossbred dairy heifers at different phases of the estrous cycle.

Parameter	Estrous Cycle Phase			
	Estrus	Metestrus	Diestrus	Proestrus
Thickness (cm)				
Left ovary	2.02±0.395	1.65±0.375	1.82±0.443	1.77±0.271
Right ovary	1.71±0.661	1.57±0.368	2.02±0.528	1.81±0.419
Length (cm)				
Left ovary	3.57±0.634	2.94±0.812	3.30±0.641	3.28±0.746
Right ovary	2.95±0.665	3.05±0.474	3.16±0.905	3.30±0.867
Area (cm^2)				
Left ovary	5.36±1.771	3.71±1.562	4.63±1.720	4.36±1.562
Right ovary	3.88±2.036	3.53±1.136	4.97±2.213	4.36±1.790
Diameter (cm)				
Left ovary	2.63±1.502	2.19±1.411	2.45±1.480	2.38±1.410
Right ovary	2.24±1.610	2.14±1.203	2.54±1.679	2.38±1.510
Perimeter (cm)				
Left ovary	10.04±1.600	8.20±1.930	9.29±1.699	9.11±1.701
Right ovary	8.20±1.934	8.13±1.190	9.18±2.333	9.00±2.121
Circularity				
Left ovary	0.65±0.076	0.67±0.063	0.65±0.074	0.64±0.054
Right ovary	0.69±0.096	0.65±0.084	0.71±0.082	0.66±0.073
Echo mean values				
Left ovary	69.42± 5.935	64.56± 6.660	67.10± 7.053	62.93± 3.767
Right ovary	68.31± 3.614^{a}	63.87± 5.557^{a}	65.56± 6.178^{a}	61.26± 3.981^{b}

Means with different superscripts among rows between periods of the estrous cycle are statistically different (P<0.05).

stroma allows the differentiation of the ovaries with the surrounding tissues. The ovarian stroma in the left ovary appeared as a heterogeneously hypoechoic structure embedded with ovoid to irregularly-shaped anechoic follicles throughout the estrous cycle (Fig. 2). Echogenicity of the right ovary varies, with the right ovary at proestrus having lower echo mean values in contrast to other periods. Throughout the estrous cycle, fluid-filled follicles, especially those > 8 mm in size, are continuously present on both ovaries in response to FSH (DesCôteaux *et al.*, 2010). Competition towards the formation of the dominant follicles from large antral follicles > 8 mm in size in this study may have resulted in compression of several follicles, causing the right ovary to appear less echogenic during proestrus.

In studies involving the ruminant ovary, the right ovary is considered to be more active in terms of a greater frequency of ovulation. More follicles measuring ≥ 5 mm was observed by Rajakoski (1960) in the right ovary of Swedish Red-and-White Breed (SRB) heifers, leading to a greater number of CL development which directly affects ovarian weight, similar to the findings in the right ovary of Zebu cattle by Dorice *et al.* (2019). Fluid-containing structures, such as ovarian follicles, are likely to have lower echotexture scores due to the poor ability of liquid to reflect soundwaves (Gonzalez-Bulnes *et al.*, 2010).

Ultrasonographic features of the follicles and corpus luteum

No significant findings (P>0.05) were observed in the dimensions (thickness, length, area, diameter, perimeter, and circularity) and echo mean values of the follicles between periods of proestrus and estrus, as opposed to the significantly higher values (P<0.05) for CL

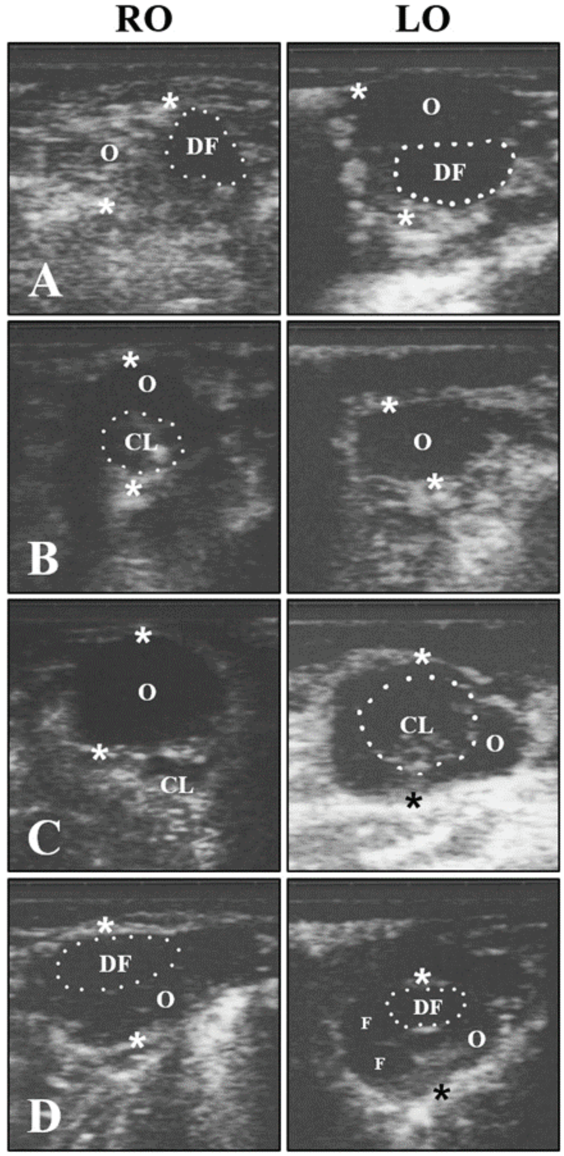

Figure 2. Representative sagittal sonograms of the right ovary (RO) and left ovary (LO) of Holstein-Sahiwal crossbred dairy heifers at estrus (A), metestrus (B), diestrus (C), and proestrus (D). The ovary (O) appears heterogeneously hypoechoic surrounded by a hyperechoic border (indicated by *). Note the presence of small ovoid anechoic follicles (F) and irregularly-shaped dominant follicles (DF) during proestrus and estrus. A developing corpus luteum (CL) in the RO can be observed during metestrus which became more distinct during diestrus.

dimensions and echo scores during diestrus (Table 4). Ovoid anechoic follicles were observed in the left and right ovaries of all heifers, with the dominant follicle being the most detectable structure during proestrus and estrus (Fig. 2). A smaller dominant follicle at estrus was observed in Holstein-Sahiwal heifers which does not coincide with the findings by DesCôteaux *et al.* (2010) using Holstein cows. In crossbred heifers, the dominant follicle was measured to be 0.67 ± 0.378 cm, smaller in size in contrast to the reported diameter of dominant follicles ranging from 11 to 16 mm, or 1.1 to 1.6 cm. Furthermore, follicles of Holstein-Sahiwal heifers were smaller compared to Holstein heifers, Brahman heifers, Nelore heifers, and Thai Native heifers with dominant follicle diameters measuring 14 to 20, 13 to 18, and 10 to 12, and 8 to 9 mm, respectively (Savio *et al.,* 1988; Rhodes *et al.,* 1995; Figueiredo *et al.,* 1997; Chasombat *et al.,* 2013).

The ovulatory follicle of Holstein-Friesian cows in a study by Sumiyoshi *et al*. (2014) revealed an estimated diameter of 14 mm on the day of CL degeneration and gradually increased to 16.50 ± 2.50 mm before ovulation. Before

Table 4. Ultrasonographic dimensions and echo mean value (Mean±SD) of dominant follicle (estrus and proestrus) and corpus luteum (diestrus) in Holstein-Sahiwal crossbred dairy heifers.

Parameter	Dominant follicle		Corpus luteum
	Estrus	Proestrus	Diestrus
Thickness (cm)	0.53±0.109[a]	0.60±0.249[a]	1.19±0.419[b]
Length (cm)	0.83±0.186[a]	0.94±0.498[a]	1.79±0.749[b]
Area (cm^2)	0.33±0.112[a]	0.51±0.483[a]	1.65±1.061[b]
Diameter (cm)	0.67±0.378[a]	0.83±0.784[a]	1.47±1.162[b]
Perimeter (cm)	2.37±0.470[a]	2.65±1.254[a]	5.18±1.829[b]
Circularity	0.73±0.092	0.76±0.055	0.71±.0.071
Echo mean value	66.77±2.930[a]	60.73±2.754[a]	94.31±10.721[b]

Means with different superscripts among rows between periods of the estrous cycle are statistically different (P<0.05).

ovulation, follicle size in Sahiwal cows was recorded to be 11.66 ± 0.60 mm on Day 20 and 11.42 ± 0.20 mm on Day 22 in animals with 2- and 3-follicular waves, respectively (Dodiyar, 2016). Zemjanis (1970) previously reported follicle size of cows in proestrus tend to range from 12 to 15 mm during Days 19 and 20 of the estrous cycle, however, follicle diameter in Holstein-Sahiwal heifers at Day 18 of the estrous cycle in this study was smaller, measuring at 0.83 ± 0.784 cm. Follicle diameter during proestrus fits the range of 7.5 to 11 mm obtained 3 days before estrus in heifers that underwent PGF luteolysis (Quirk *et al.*, 1986). There was no significant difference on the circularity between dominant follicles during estrus and proestrus. Likewise, there was no significant difference between the dominant follicles during estrus and proestrus, and the CL during diestrus since all the values obtained were less than 1.0.

Although not significant, a shorter estrous cycle may have affected the size of the dominant follicle between the phases of estrus and proestrus. An inverse relationship regarding the size of the dominant follicle and the duration of proestrus was observed by Sirois and Fortune (1988). Low circulating progesterone levels during this stage enhanced follicular development, resulting in larger follicle size, and influencing the duration of proestrus in Holstein-Sahiwal heifers. Measurements obtained during this period may have overlapped with the start of the estrus phase of the succeeding cycle. Furthermore, despite the presentation of signs of heat (clear mucous discharge, swollen vulva, and color changes of heat detector patches) on Day 0 in all heifers, follicle measurements may have been obtained during early estrus where peak estrogen levels have not been attained to induce GnRH and LH pulses which supports the maturation and maximum development of the dominant ovulatory follicle (Hopper, 2014). Obtaining the size of the dominant follicle during estrus from two successive ovulations can reduce discrepancies in follicle size.

Inconsistencies can be also attributed to the differences in the number of follicular wave cycles as 2-follicular wave cycles have a shorter number of days in a cycle, resulting in shorter estrus detection in contrast to heifers with 3-follicular wave cycles (Chasombat *et al.,* 2013). Ginther (1993) reported the ambiguity regarding overlapping follicle diameter profiles in cases wherein two successive dominant follicles developed and formed on the same ovary, as well as subordinate follicles from subsequent waves merging with regressing follicles. Obtaining follicle size based on the volume of the follicular fluid can prevent underestimation of follicles measuring 6 to 8 mm, and overestimation of follicles ≥ 12 mm (Spicer *et al.*, 1987).

Lower echo values for the dominant follicle detected during estrus and proestrus were observed in contrast to the CL due to the presence of follicular fluid. The follicle appeared as an ovoid anechoic structure either partially or fully surrounded by an echogenic border in agreement with the findings by Quirk *et al.* (1986), Assey *et al.* (1993), Ribadu and Nakao

(1999), and DesCôteaux *et al.* (2010). Tom *et al.* (1998) and Honaramooz *et al.* (2004) emphasized that changes in the lifespan, vascularity of the theca interna, follicle viability, steroid synthesis, and rate of proliferation of ovarian follicles can significantly cause differences in ultrasonographic pixel values. Echogenicity of the dominant follicle varies, with high follicular wall scores and a lower, heterogenous antrum score observed during the growth phase (ovulation to Day 6) before dropping during the early static phase (Tom *et al.*, 1998).

Table 4 shows the ultrasonographic dimensions and echo mean values of dominant follicle during estrus and proestrus and corpus luteum during diestrus. Five of 10 dominant follicles were detected on the right ovary, and also five of 10 dominant follicles were detected on the left ovary during estrus. In contrast to the findings by Zemjanis (1970) and Hansen *et al.* (2000), the diameter of the CL during diestrus in this study does not coincide with the previous measurement of 20 to 30 mm. CL diameter obtained on Day 11 after ovulation in both ovaries was 1.47 ± 1.162 cm, falling within the CL measurement range of 13.55 ± 0.17 to 15.14 ± 0.14 mm in Thai Native heifers from Days 9 to 11 of the estrous cycle (Chasombat *et al.*, 2013). In Holstein-Friesian cows, the maximum CL size measured by Herzog *et al.* (2010) at peak progesterone concentration was 3.7 ± 0.1 cm^2 for compact CLs and 5.2 ± 0.2 cm^2 for cavitary CLs at Day 14 post-ovulation, relatively larger compared to the obtained CL area of 1.65 ± 1.061 cm^2 from both ovaries of Holstein-Sahiwal heifers. Likewise, CL thickness and length in Holstein-Sahiwal heifers used were smaller compared to the height and length of the CL of pregnancy in Holstein-Sahiwal cows that measured from 18.0 ± 6.43 to 21.1 ± 4.07 mm and 17.5 ± 4.92 and 26.3 ± 4.16 mm, respectively (De Ramos *et al.*, 2008). Similarities in the CL size during its static and regression phases can be considered as one of the constraints in determining the phase of the estrous cycle by mainly relying on the sonographic image of the CL (Herzog *et al.,* 2010).

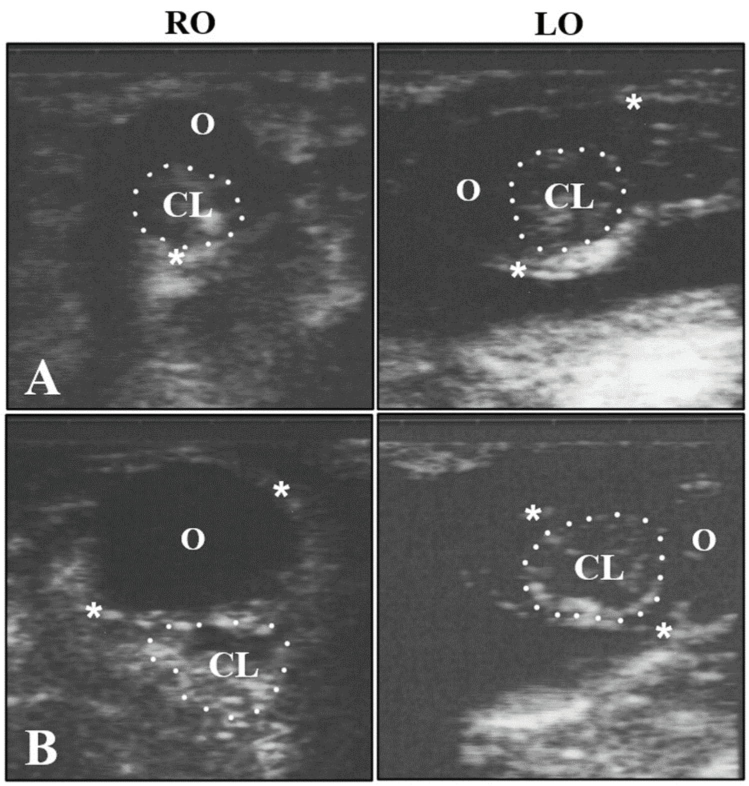

Figure 3. Representative sagittal sonograms of the right ovary (RO) and left ovary (LO) of Holstein-Sahiwal crossbred dairy heifers at metestrus (A) and diestrus (B). The ovary (O) appears heterogeneously hypoechoic surrounded by a hyperechoic border (indicated by *). The homogenously hypoechoic corpus luteum (CL) during metestrus and diestrus appeared as a granular structure with no distinct outline.

The CL appeared as a more echogenic structure in contrast to the follicles with lower echo scores (Fig. 3). The CL was detected during diestrus on the right ovary of five out of 10 (50 %) heifers and also the left ovary of five out of 10 (50 %) heifers. These are the same dominant follicles observed during estrus that ovulated. This is in contrast to previous findings that the right ovary is more active than the left. The ultrasonographic appearance of the CL during diestrus appeared as an irregularly-shaped, homogenously hypoechoic granular structure with no distinct outline. Echogenicity of the CL agrees with the general description of Assey *et al.* (1993), Ribadu and Nakao (1999), and Kähn and Volkmann (2004) but slightly differ due to the absence of an echogenic border or demarcation. Distinguishing the CL as either compact or cavitary may be difficult to perform using the available sonograms due to the absence of obvious anechoic cavities with a diameter ranging from 3 to 10 mm, and the inability to distinguish luteal tissues on the ovary (Kastelic *et al.*, 1990; Assey *et al.*, 1993; DesCôteaux *et al.*, 2010).

Findings of this study presented the ultrasonographic measurements of the uterus, ovaries, and associated structures in crossbred dairy heifers. The physiological response associated with the estrous cycle reflected changes in the appearance and dimensions of the above-mentioned organs, particularly the left uterine horn, dominant follicle, and corpus luteum. Similarities in the characteristics and echogenicity of the uterus, ovaries, dominant follicle, and CL during ultrasound examination were observed when compared with previous studies using purebred animals. The inclusion of hormonal assays for detection of the changes in plasma luteinizing hormone, estrogen, and progesterone concentrations, and the potential application of Doppler ultrasound to measure uterine and ovarian arterial blood perfusion as indicators for changes in the physiological status of the reproductive tract can aid in future researches involving the bovine estrous cycle. Ultrasound examination can also be repeated using crossbred heifers subjected under different environmental conditions (e.g. subtropical versus temperate) to investigate differences in uterine and ovarian dimensions.

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STATEMENT ON COMPETING INTEREST

The authors have no competing interest to declare.

AUTHOR'S CONTRIBUTION

Author KYYP contributed to the development of the methodology, provided resources, performed the experiment, collected data, applied statistical techniques to analyze the data, wrote the original draft, and performed review and editing of the manuscript. Authors AMGAP and AAR conceptualized the study, designed the methodology, provided resources, supervised the research activity, reviewed and edited the manuscript. Author AMGAP also did the statistical analysis and interpretation of the data collected.

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