Preovulatory follicle characteristics and oocyte competence in repeat breeder dairy cows

P. Sood,*† M. Zachut,* I. Dekel,* H. Dube,* S. Jacoby,* and U. Moallem*¹

*Department of Ruminant Science, Institute of Animal Sciences, the Volcani Center, 68 HaMaccabim Road, Rishon LeZion 75359, Israel

†Department of Veterinary Gynecology and Obstetrics, DGCN College of Veterinary and Animal Sciences, Himachal Pradesh Agricultural University, Palampur 176 062 Himachal Pradesh, India

ABSTRACT

The varied and elusive etiology of repeat breeding (RB) in dairy cows necessitates evaluation of oocytes and follicles, which have not previously been assessed together. Accordingly, we evaluated characteristics of preovulatory follicles and the competence of oocytes in control (CTL) and RB cows. The estrous cycles of 35 cows (18 CTL and 17 RB) were synchronized using PGF_{2α} and estrus detection. Cows with a corpus luteum were treated with PGF_{2α} and, 14 to 15 d after a visible behavioral estrus, they were administered a second PGF_{2α}, followed 48 h later by follicular fluid (FF) aspiration of the preovulatory follicles. Estradiol (E₂)-active preovulatory follicles did not differ in diameter between the 2 groups of cows. However, FF of RB cows had higher E₂ concentrations than that of CTL cows: 1,854.9 and 1,073.6 ng/mL, respectively, but similar androstenedione and progesterone concentrations. In the second part of the study, 14 consecutive ovum pick-ups (OPU) were performed in 5 CTL and 5 RB cows, at 3- to 4-d intervals. The RB and CTL cows did not differ in average numbers of follicles available per cow per session (7.1 and 7.3, respectively), oocyte recovery rates (42.2 and 44.1%, respectively), or cleavage rates (57.6 and 63.4%, respectively), but blastocyst production was markedly less in RB than in CTL cows (12.5 and 29.2%, respectively). We conclude that part of the RB cows' etiology occurs at an earlier phase of folliculogenesis, thereby impairing oocyte competence, and subsequently reducing the probability of normal fertilization, which diminish embryo vitality and development.

Key words: repeat breeder dairy cow, preovulatory follicle, oocyte competence

INTRODUCTION

The incidence of repeat breeding (RB) in some dairy cattle herds is as high as 24% (Yusuf et al., 2010); in Israel, the frequency of RB in multiparous dairy cows in recent years has been ~30% (Israeli Dairy Herdbooks; Israel Cattle Breeders' Association, 2014, 2015). Among other costs, the RB phenomenon results in loss of genetic gain and increased culling rates (Bartlett et al., 1986). Reproduction in cows depends on many factors and involves tight coordination of numerous events that occur at various points between the higher brain centers and the ovarian compartment. Fertilization failure (Graden et al., 1968) and embryo mortality (Gustafsson, 1985) are the inherent mechanisms that restrict reproduction under various adverse conditions, of which RB is a pertinent example. There are several documented reasons for subnormal fertility in RB cows; some investigations found endocrine and ovulatory disturbances to be the preponderant reasons (Båge et al., 2002; Saumande and Humbolt, 2005; Bloch et al., 2006; Sood et al., 2015). However, these can be diagnosed from clinical manifestations (e.g., prolonged estrus) or endocrinological assessments, and treated accordingly (López-Gatius et al., 2005). Nevertheless, other groups of RB cows had normal endocrine and ovulation status, normal genital organs (Sood et al., 2015), and a uterine environment that was favorable for pregnancy (Tanabe et al., 1985). In such cases, determining the exact reason for RB remains a serious challenge, and the etiology remains obscure in 37.8% of the RB cows (Perez-Marin and Espana, 2007). Thus, the causes of RB lie beyond endocrine or ovulatory perturbations, and we assumed that RB might be determined much earlier, during the course of oocyte development. In a previous study, RB cows yielded substantially increased numbers of morphologically deviated and degenerated 7-d-old embryos, which were collected nonsurgically from their uterus (Gustafsson, 1985). Other studies revealed excess inferior-quality oocytes in RB cows (Gustafsson and Emanuelson, 2002; Båge et al., 2003; Kurykin et al., 2011). Ferreira et al. (2011) found no differences

Received April 2, 2017.

Accepted August 1, 2017.

¹Corresponding author: uzim@volcani.agri.gov.il

in quality or cleavage rate between oocytes retrieved from RB cows and those from cows in peak lactation, but they observed reduced blastocyst production in the RB cows; however, Ferreira et al. (2011) performed their study after involvement of exogenous hormones in the synchronization protocol, which might influence oocyte quality or competence (Blondin et al., 2012). The oocyte shares a close and dynamic relationship with the antral follicular fluid (FF), which constitutes a specialized microenvironment specifically suited to the needs of the developing oocyte. Composition of the FF, especially the steroids, can influence follicle health and oocyte quality (Wehrman et al., 1993; Revelli et al., 2009). Aardema et al. (2013) examined the association, in superstimulated healthy heifers, between steroid concentrations in preovulatory follicles and oocyte developmental competence: they found differences in the criteria for selection of cumulus-oocyte complexes between superstimulated heifers and nonstimulated cows, and this finding highlights the need to examine the RB phenomenon in nonstimulated cows.

Zachut et al. (2010) investigated the FF steroidal environment and oocyte competence in cows subjected to dietary changes, and Roth et al. (2008) did so for those in various stages of lactation, but neither group addressed RB cows. Therefore, the aim of the present study was to delineate the RB mechanisms, per se, in unstimulated RB cows.

MATERIALS AND METHODS

Cows and Diet

All procedures used in the present study were approved by the Volcani Center Animal Care Committee. The animals used were primiparous and multiparous lactating Israeli Holstein cows, housed and maintained at the Volcani Center Experimental Farm (Rishon Lezion, Israel). All the selected cows were normal cyclic, with normal estrus duration, and before the start of

the study had no history of dystocia, retained placenta, or metritis. Also, we ruled out occurrence of abnormal genital discharge and of pathological abnormalities of the reproductive tract, including cystic ovaries, by performing 2 successive examinations separated by 10 to 11 d, before the study commenced; using an Aquila 5-MHz linear array transducer (Pie Medical, Maastricht, the Netherlands). The other exclusion criteria were severe mastitis, lameness, or severe digestive disorders during the current lactation.

The cows were grouped into 2 categories: control (CTL) and RB. The CTL cows were >60 d in lactation, with a range of 62 to 133 d, cycling, and not inseminated. A cow was considered RB if it exhibited no clinically detected abnormality and did not become pregnant after at least 4 successive AI during spontaneous estrus, in the current lactation, with normal intervals between inseminations. Duration of estrus was recorded by means of H-tag collar-mounted tags for neck movements (SCR Engineers, Hadarim, Netanya, Israel) and pedometers (Afifarm System, SAE Afikim, Israel).

Forty cows were investigated during 2 successive years. In the first year, the study involved 25 cows, 13 CTL and 12 RB; we assigned 20 cows (10 from each group) to preovulatory follicular aspiration and 10 cows (5 from each group) to ovum pick-up (OPU). Five cows (2 CTL and 3 RB) were subjected to both procedures, and 5 cows (3 CTL and 2 RB) were subjected only to OPU. In the second year, 15 cows (8 CTL and 7 RB) were assigned only to preovulatory FF aspiration; see Table 1 for distribution of cows among both procedures. We conducted the experiment during the same season in each year (late winter to early spring) to avoid heat stress effects.

The cows were fed according to NRC (2001) recommendations and housed together in a covered loose pen with an adjacent outside yard. The animals were milked 3 times/d, and milk yields and BW were automatically recorded daily with an automatic Afifarm System

Table 1. Distribution of cows for preovulatory follicle aspiration and ovum pick-up (OPU) procedures in both years¹

Procedure	First year		Second year		Total cows
	CTL	RB	CTL	RB	
Preovulatory follicle aspiration only	8	7	8	7	30
Preovulatory follicle aspiration + OPU	2	3	0	0	5
OPU only	3	2	0	0	5
Total preovulatory follicle aspiration	10	10	8	7	35
Total OPU	5	5	0	0	10
Total cows	13	12	8	7	40

¹CTL = control cows; RB = repeat breeder cows.

(SAE Afikim). A single technician determined the BCS (Edmonson et al., 1989) in all cows at the beginning of each procedure.

Follicular Fluid Aspiration

Estrous Cycle Synchronization and Blood Sampling Schedule. Follicular fluid aspiration from preovulatory follicles was conducted during 2 successive years—20 cows in the first year and 15 in the second year. In each year, the cows were designated as CTL or RB, as described above. In total, we considered 18 cows as CTL and 17 as RB. After confirming the presence of a corpus luteum (CL) in each cow, they were treated intramuscularly with a $\text{PGF}_{2\alpha}$ analog, cloprostenol sodium (Estroplan, Parnell Living Science, Alexandria, NSW, Australia) at a dose of 500 μg per cow. The cows were examined for signs of estrus and, at 14 to 15 d after a visible behavioral estrus, they received another $\text{PGF}_{2\alpha}$ injection, followed after 48 h by FF aspiration. A schematic representation of follicle aspiration protocol is presented in Figure 1.

Jugular venous blood samples were collected in lithium heparin-coated vacuum tubes (Becton Dickinson Systems, Cowley, UK) on the day of the second $\text{PGF}_{2\alpha}$ treatment and 48 h later; that is, in parallel with aspiration of preovulatory follicles, to verify that the cows were in the expected stage of the estrous cycle. Plasma was harvested after centrifugation for 15 min at $3,000 \times g$ and stored at -32°C , pending analysis of progesterone (P_4).

FF Aspiration Procedure. Forty-eight hours after the second $\text{PGF}_{2\alpha}$ administration, we performed FF aspiration as described by Moallem et al. (1999). Briefly, the cows were sedated with intramuscular administration of 1 mL of 2% Rompun (xylazine base, 20 mg/mL; XYL-M2 Veterinary, VMD, Arendonk, Belgium) and given local anesthesia in the form of 5 mL of 2% lidocaine HCl (200 mg per 10 mL; Esracain 2%; Rafa Laboratories, Jerusalem, Israel) injected epidurally between the last sacral and first caudal vertebrae, in the sacro-coccygeal space. Ovaries were monitored, and follicles ≥ 7 mm in diameter were aspirated separately. The FF was aspirated with the aid of an ultrasound scanner (Pie Medical) connected to a 7.5-MHz vaginal sector transducer equipped with a needle guide and connected to an MP86 suction pump (Biometra, Goettingen, Germany) set at a flow rate of 25 to 30 mL/min. The needles used were 18 gauge and changed between follicles. After collection, the FF was centrifuged for 15 min at $3,000 \times g$, the sediment (including granulosa cells) was separated from the fluids, and both fractions were frozen at -32°C pending analysis.

Figure 1. Schematic representation of follicle aspiration protocol. Cows received $\text{PGF}_{2\alpha}$ analog on d -2; then, 14 to 15 d after behavioral estrus (d 0), they received another $\text{PGF}_{2\alpha}$ dose, and 48 h later follicular fluid from the large follicles was aspirated.

OPU Procedure and Oocyte Collection. The OPU procedure was performed on 5 CTL and 5 RB cows, twice weekly, on Monday and Thursday, for 7 consecutive weeks (a total of 14 OPU sessions). The number of visible 3- to 6-mm-diameter follicles was recorded and all such follicles from each ovary were aspirated into a single 50-mL tube containing HEPES-Tyrodé's lactate solution medium (Sigma-Aldrich Israel, Rehovot, Israel) supplemented with antibiotics and 0.008% heparin (Sigma-Aldrich Israel), in 0.4% BSA (Sigma-Aldrich Israel). The tubes containing aspirated follicular material were transferred to an adjacent laboratory for recovery and morphological examination of cumulus-oocyte complexes (COC). According to the number of layers of cumulus surrounding the oocytes and their cytoplasmic consistency, we classified the COC into 4 categories as described by de Loos et al. (1989): grade 1: spherical, symmetrical, and intact oocytes of uniform size, color, and texture, entirely surrounded by 3 to 5 compact layers of cumulus cells; grade 2: incomplete cumulus layer, oocyte partially denuded; grade 3: cumulus expanded and partially degenerated, oocyte denuded; and grade 4: totally degenerated cumulus and oocyte.

Oocyte Maturation. The procedure for in vitro oocyte maturation (IVM) and fertilization (IVF) was as described by Zachut et al. (2010). Briefly, after recovering and grading the oocytes, we washed all grade 1 and grade 2 COC with K-SIWB oocyte wash buffer (Cook, Sydney, Australia) and transferred them separately for each animal into 4-well culture multi-dishes (Nunc, Roskilde, Denmark) containing 500 μL of TCM-199 maturation medium (Sigma-Aldrich Israel), and incubated them for 22 h at 38.5°C in humidified air containing 5% CO_2 .

IVF. After maturation, we washed the COC once in HEPES-Tyrodé's albumin, lactate, and pyruvate (Sigma-Aldrich Israel), mechanically denuded them of cumulus cells with a pipette, and placed them in their respective groups in 4-well plates, with approximately 10 oocytes/well. Each well contained 500 μL of In Vi-

tro Fert (Cook) with 0.0005% heparin. Semen from a single Israeli-Holstein bull of proven fertility was used throughout the study. Before use, the frozen semen straws were thawed for 30 s in a water bath at 35°C. Then, the semen was placed on a 90 to 45% Percoll gradient prepared with sperm wash medium (modified Tyrode medium) and centrifuged at $320 \times g$ for 10 min to isolate the motile sperm and to remove the diluents and seminal plasma. The aspirated sperm pellet was evaluated for motility and concentration. Each fertilization drop received a final concentration of 1×10^6 spermatozoa/mL. After addition of sperm, 20 μ L of a solution of 0.5 mM penicillamine (Sigma-Aldrich Israel), 0.25 mM hypotaurine (Sigma-Aldrich Israel), and 25 μ M epinephrine (Sigma-Aldrich Israel) in 0.9% (wt/vol) NaCl solution was added to each well. The sperm and COC were co-incubated for 18 h at 38.5°C in humidified air containing 5% CO₂, 5% O₂, and 90% N₂. Presumptive zygotes were washed once in In Vitro Cleave (Cook) and then placed into 50- μ L culture drops of In Vitro Cleave, with 5 to 10 oocytes/drop. The oocytes were cultured at 38.5°C in humidified air containing 5% O₂, 5% CO₂, and 90% N₂ for another 22 h. We then calculated the proportional cleavage rate; that is, the total number of oocytes that cleaved divided by total number of viable oocytes. After a further 6 d, on completion of the second feeding, we determined the blastocyst rate; that is, the total number of blastocysts divided by total number of viable oocytes.

Chemical Analysis

The FF of all aspirated preovulatory follicles ($n = 66$) was assessed for concentrations of P₄ (Diagnostic Products, Los Angeles, CA), estrogen (E₂; Diagnostic

Products), and androstenedione (A₄; Diagnostic Systems Laboratories, Webster, TX). All FF samples were diluted 100-, 500-, or 30-fold for P₄, E₂, and A₄ assays, respectively, so that the assay results matched the detection ranges. We also determined P₄ concentrations in blood plasma. The minimal detectable concentrations of P₄, E₂, and A₄ were 0.2, 20, and 0.1 ng/mL, respectively. The intra- and interassay coefficients of variation for the P₄, E₂, and A₄ assays were 9.2, 4.1, and 6.1%; and 8.5, 3.6, and 4.5%, respectively. We considered follicles with E₂:P₄ ratios >1 to be E₂-active (Ireland and Roche, 1982) and subjected them to further analysis and evaluation.

Statistical Analysis

The hormone concentrations in FF were analyzed with the general linear models (GLM) procedure of SAS, version 9.2 (SAS Institute, 2002). The effect of cluster was included in the model.

Number of follicles, oocyte numbers and grading, and oocyte recovery rate were calculated for each cow, and the results were analyzed with Proc Mixed (SAS Institute, 2002). The model included effects of group, cow (nested in group), session, and group $\times$ session interaction.

For matured oocytes, cleavage rate, blastocyst number and rate, and session were considered as random effect, and data were analyzed with the Proc Mixed procedure of SAS; group, session, and group $\times$ session interaction included in the model.

Least squares means and adjusted SEM are presented in Tables 1 and 2. A level of $P < 0.05$ is accepted as statistically significant unless otherwise stated, and tendencies are reported at $0.05 < P < 0.10$.

Table 2. Concentrations of progesterone, androstenedione, and estradiol in estrogen (E₂)-active follicles aspirated from control (CTL) or repeat breeder (RB) cows¹

Item	Group		SEM	P-value
	CTL	RB		
Cows, no.	17	17		
Follicles aspirated, no.	36	30		
Atretic follicles, no.	17	13		
E ₂ -active follicles, no.	19	17		
Diameter, mm	15.4	15.9	1.0	0.69
Volume, mL	2.32	2.56	0.51	0.75
Progesterone, ng/mL	118.5	119.3	25	0.98
Androstenedione, ng/mL	275.0	317.8	90.0	0.75
Estradiol, ng/mL	1,073.6	1,854.9	138.2	0.0005
Estradiol:progesterone ratio	12.7	31.6	6.5	0.05
Total estradiol content, μ g	2.7	5.2	0.98	0.1
Total progesterone content, μ g	0.35	0.45	0.21	0.74
Total androstenedione content, μ g	1.07	1.10	0.49	0.96

¹Preovulatory follicles were aspirated 48 h after prostaglandin injection from 17 CTL and 17 RB cows.

Table 3. Number of follicles aspirated and of oocytes collected by ovum pick-up (OPU) along with their in vitro maturation¹

Item	Group		SEM	<i>P</i> <
	CTL	RB		
Total oocytes aspirated, no.	520	486	—	—
Follicles aspirated/cow per session, no.	7.3	7.1	0.39	0.77
Oocytes recovered/cow per session, no.	3.21	3.1	0.25	0.70
Grade 1 oocytes rate, %	28.4	21.3	3.6	0.21
Recovery rate, %	44.1	42.2	0.03	0.68
No. of oocytes/cow per session chosen for IVM + IVF	2.3	2.3	0.25	1.0
No. of cleaved oocytes/session	7.1	6.5	0.9	0.63
Cleavage rate, %	63.4	57.6	3.3	0.23
No. of blastocysts/session	1.81	0.72	0.21	0.002
Rate of blastocysts from oocytes cleaved, %	29.2	12.5	3.2	0.002

¹The OPU procedure was performed twice weekly on 5 control (CTL) and 5 repeat breeder (RB) cows in 14 OPU sessions; all visible 3- to 6-mm-diameter follicles were aspirated. The oocytes were categorized, and grade 1 and 2 oocytes were subjected to in vitro maturation (IVM) and fertilization (IVF) procedures.

RESULTS

Preovulatory Follicular Fluid

The general data from cows subjected to preovulatory FF aspirations on the day of the first PGF_{2α} treatment, averaged over both years for the CTL and RB groups, respectively (expressed as mean ± SD) were as follows: milk yield, 49.6 ± 8.0 and 34.6 ± 7.6 kg/d (*P* = 0.0001); 4% FCM, 45.6 ± 6.2 and 32.8 ± 6.8 kg/d (*P* = 0.0001); parity, 2.8 ± 1.3 and 2.2 ± 1.3 (*P* = 0.16); BW, 635.2 ± 62.7 and 695.2 ± 76.1 kg (*P* = 0.02); DIM, 90.2 ± 27.3 and 343.2 ± 102.3 d (*P* = 0.0001); and BCS, 2.8 ± 0.2 and 2.9 ± 0.1 (*P* = 0.32). The number of AI in the RB cows subjected to FF aspiration ranged from 4 to 10, and averaged 7.1 ± 1.7.

The characteristics of preovulatory follicles and FF are presented for comparison between CTL and RB cows in Table 2. We excluded 1 CTL cow from the analysis because of a large follicle (32 mm in diameter) on 1 ovary, which may be regarded as a cystic follicle. In total, we aspirated preovulatory follicles from 17 CTL and 17 RB cows in 2 clusters. The average diameter and volume of the preovulatory follicles did not differ between the groups (Table 2). We defined 19 out of 36 (52.3%) follicles aspirated in the CTL group and 17 out of 30 (56.7%) in the RB group as E₂-active preovulatory follicles and the remainder as atretic follicles; that is, E₂-inactive. The steroidogenic environments of the respective groups contained similar P₄ and A₄ concentrations, whereas the E₂ concentration was 73% higher in the RB than in the CTL cows (*P* < 0.0005). Also, the E₂:P₄ ratio was 42.1% greater in RB than in CTL (*P* < 0.05); the E₂ content, expressed as micrograms per follicle, was 93% greater in RB than in CTL cows (*P* < 0.1); we detected no differences between the groups in P₄ and A₄ contents.

Peripheral blood plasma P₄ concentrations in CTL and RB cows on the day of the second PGF_{2α} administration were similar: 6.45 ± 0.62 and 7.38 ± 1.16 ng/mL, respectively; and P₄ concentrations 48 h later, at aspiration, were 0.31 ± 0.19 and 0.37 ± 0.10 ng/mL, respectively. Blood P₄ concentrations depended on the estrous cycle synchronization protocol and confirmed that cows from the respective groups were at the expected stage of the cycle.

OPU Results

The general data on cows subjected to OPU, averaged over CTL and RB groups, respectively (expressed as mean ± SD) were as follows: milk yield, 48.2 ± 5.6 and 39.6 ± 7.4 kg/d (*P* = 0.07); 4% FCM, 47.6 ± 6.1 and 35.0 ± 7.1 kg/d (*P* = 0.02); parity, 2.0 ± 1.0 and 2.0 ± 1.0 (*P* = 1.0); BW, 638.0 ± 65.3 and 654.0 ± 116.5 kg (*P* = 0.79); DIM, 70.6 ± 23.4 and 366.6 ± 102.0 d (*P* = 0.0002); and BCS, 2.9 ± 0.2 and 3.1 ± 0.2 (*P* = 0.30). The average number of AI in the RB cows subjected to OPU ranged from 5 to 10 and averaged 6.8 ± 1.9 (Table 3).

We collected 520 and 486 oocytes from CTL and RB cows, respectively, during 14 OPU sessions. The number of follicles available for OPU was 7.3 ± 2.5 and 7.1 ± 3.1 per cow/session, respectively, and did not differ between CTL and RB cows. Similarly, the recovery rates of oocytes and the numbers of oocytes selected for IVM and IVF procedures did not differ between the 2 groups. The cleavage rate (%) and the number of cleaved oocytes per session also did not differ between groups. The number of blastocysts per session, 0.72 and 1.81, respectively, and percentages of blastocysts obtained from oocytes that cleaved, 12.5 and 29.2%,

respectively, were markedly lower ($P < 0.002$) in the RB cows than in the CTL cows.

DISCUSSION

The complex etiology of RB requires careful evaluation to identify the reason for reproduction failure, especially in cows without diagnosed clinical signs. In the present study, we evaluated preovulatory follicle characteristics and oocyte competence, and the findings indicate diminished oocyte competence in early stages of development in RB cows, which may impair the subsequent vitality and development of the embryos in these animals.

Preovulatory Follicle Characteristics

In the present study, the size of preovulatory E_2 -active follicles in the RB and CTL cows were similar. However, in a study conducted in heifers, Båge et al. (2002) found that the median diameter of preovulatory follicles was significantly greater in RB animals than in virgin heifers, at 16 and 14 mm, respectively. Earlier studies have found wide variability in the diameter of the ovulatory follicle in RB cows, ranging from 12 to 17.8 mm and implying that follicle size does not cause repeat breeding (Dovensky et al., 2000; Perez et al., 2003). Furthermore, the average follicle diameter in both groups in the current study was >15 mm, and it is known that follicle size does not limit oocyte competence once follicles exceed 8 mm in diameter (Hyttel et al., 1997; Kauffold et al., 2005).

Hormones in the cows' FF influence oocyte growth and differentiation, directly through genomic and nongenomic actions, and indirectly through somatic cell investments in the follicle (Revelli et al., 2009); the oocyte also produces factors that regulate follicular steroidogenesis (Vanderhyden et al., 1993). Steroidal output of P_4 in the FF is required for final maturation of the oocytes, ovulation, and subsequent embryo survival (Christian and Moenter, 2010; Lynch et al., 2010), but excessive concentrations of P_4 in the FF are detrimental (Ben-Rafael et al., 1987). However, in the present study, our finding of similar P_4 concentrations in the FF of CTL and RB cows excluded this possible effect on oocyte competence.

In the present study, we found similar P_4 concentrations in the blood of CTL and RB cows at aspiration time, in agreement with our previous findings (Sood et al., 2015). However, earlier studies attributed the reproductive failure in RB cows to increased suprabasal progesterone concentrations during estrus (Båge et al.,

2002, 2003), which we did not find in our present or previous studies (Sood et al., 2015).

Androstenedione acts as a precursor that is aromatized to form E_2 (Walters et al., 2008); as follicle size increases, E_2 concentration in the FF continues to increase, whereas the concentration of A_4 decreases (Henderson et al., 1982). A lower $A_4:E_2$ ratio in the FF therefore indicates that increased A_4 contributes to production of E_2 and vice versa (Henderson et al., 1982). Increased A_4 , either absolutely or relative to E_2 (Revelli et al., 2009), is linked to lower oocyte quality or reduced postfertilization cleavage (Uehara et al., 1985). However, in the present study, no differences between groups were found in concentration or content of A_4 or in the $A_4:E_2$ ratio, which was 0.24 and 0.21 in the CTL and RB cows, respectively.

In the present study, the most prominent difference between FF steroidal concentrations in the respective cow groups was that E_2 concentrations were 73% higher in RB than in CTL cows. Studies have found that FF E_2 concentrations elicited varied effects on follicles and oocytes: some reported that predominant estrogenic environment in the FF elicited anti-atresia effects and good follicular growth, and enhanced cytoplasmic maturation of the oocytes (Tesarik and Mendoza, 1997), whereas other studies found no beneficial effects on oocytes of higher E_2 (Messinis and Templeton 1987; Dawson et al., 2006). Increased E_2 in the FF, which probably led to increased circulating E_2 around periestrus in the RB cows (Sood et al., 2015), increased the likelihood of achieving pregnancy (Kreiner et al., 1987; Tarlatzis et al., 1993; Teissier et al., 2000) through improvements in sperm transport, fertilization (Hawk, 1983), and embryo quality and viability (Atkins et al., 2013; Jinks et al., 2013). However, increased circulating E_2 , derived from intrafollicular E_2 , might create a favorable uterine environment in RB cows, as was reported previously (Tanabe et al., 1985); however, excessively increased intramolecular E_2 concentrations and $E_2:P_4$ ratios might, in turn, result in a relatively more advanced stage of oocyte maturation, and thereby compromise oocyte competence (Kreiner et al., 1987; Tarlatzis et al., 1993; Teissier et al., 2000).

Previous studies found a strong correlation between the diameter of the dominant follicle and the intrafollicular concentration of E_2 during the pre-ovulatory period (Ireland and Roche, 1982; Kruip and Dieleman 1985). Aardema et al. (2013) did not find follicular diameter to be a good predictor of oocyte quality. Luteinizing hormone acts on granulosa cells to aromatize androgens to E_2 in follicles selected for further development. In light of several studies that indicated an attenuated periestrual profile of LH in the circulation

of RB cows (Saumande and Humbolt, 2005; Bloch et al., 2006; Sood et al., 2015), the increase in E_2 may not be attributed to increased LH secretion; instead, differences in expression of aromatase enzyme, in number of granulosa and theca cells, or altered LH sensitivity of the cellular investments in the follicle (Webb and Campbell, 2007) could account for the higher E_2 in RB cows.

Interestingly, oocytes also secrete factors that regulate E_2 production in the follicle. In mice, FSH and testosterone stimuli elicited production of almost twice as much E_2 from cultured granulosa cells containing oocytes than from similar numbers of granulosa cells without oocytes. (Vanderhyden et al., 1993). Thus, variations in oocyte involvement in regulating E_2 production might be another factor that affects E_2 production in RB cows.

OPU Results

Follicle Number at OPU. Van der Schans et al. (1991) and Li et al. (2007) found that a protocol involving twice-weekly OPU at 3- to 4-d intervals uncoupled the follicular wave from the estrous cycle and increased the wave frequency without affecting the number of recruited follicles. In the present study, the average number of follicles available for aspiration was similar between the 2 groups, as Båge et al. (2002) also found when they compared normal and RB heifers. Jimenez-Krassel et al. (2009) and Mossa et al. (2010a,b) found that follicular counts in specific individuals were highly repeatable, similar to the current study, in which the effect of cow on follicle number was significant at $P < 0.05$. In addition, Mossa et al. (2012) found that cows with higher follicle counts had 3.34 times greater probability of becoming pregnant than those with fewer follicles, and Jimenez-Krassel et al. (2009) reported that cows with lower follicle counts had reduced uterine endometrial thickness and diminished likelihood of pregnancy. In the present study, the similarity between groups in their follicle counts at OPU also ruled out this parameter as a factor affecting fertility of RB cows.

Oocyte Recovery. The average numbers of recovered oocytes were similar between RB and CTL cows, at 3.1 and 3.2 oocytes/cow per session, respectively, and were close to the value of 3.05 found in the control cows of our previous study (Zachut et al., 2010). Båge et al. (2002) reported a lower oocyte recovery rate in RB cows than in virgin heifers, 55 and 62%, respectively, and suggested that it was linked to poor oocyte quality. Although the number of oocytes we chose for IVF in the present study did not differ between RB and CTL cows, several previous studies found 30 to 93.3% more

abnormal oocytes in RB than in normal cows (Båge et al., 2002; Kurykin et al., 2011).

Variation has been noted in the yield of abnormal oocytes among RB cows: some but not all of them consistently produce abnormal oocytes (Båge et al., 2002; Kurykin et al., 2011). However, this was not the case in the present study, in which no RB cow produced exceptionally higher or lower number of abnormal oocytes, as indicated by lack of significant interaction between cows, sessions, and oocyte quality. Thus, occurrence of variation among different studies in the relation between number of normal and abnormal oocytes highlights the multiplicity and intricate etiology of RB.

Cleavage and Blastocyst Rates. Båge et al. (2002) reported a lower cleavage rate in RB cows than in normal cows and virgin heifers, in contrast to our current findings and those of Ferreira et al. (2011). However, the blastocyst rates in the present study were 12.5 and 29.2% in RB and CTL cows, respectively; Ferreira et al. (2011) also found low competence in oocytes retrieved from synchronized RB cows. Oocyte losses in the present study were nearly 34.2 and 45.1% between cleavage and blastocyst formation in the CTL and RB groups, respectively. This finding is consistent with that of Lonergan et al. (2001), who found that 30 to 40% of cleaved oocytes failed to yield blastocysts; they attributed this to the intrinsic quality of the oocytes. In the present study, the considerably lower blastocyst formation rate in the RB group could be due to compromised oocyte competence; Assey et al. (1994) and Hyttel et al. (1997) observed that a series of ultrastructural and molecular changes occurred during oocyte development within the follicle, which could affect oocyte competence. Interestingly, in a previous study, Båge et al. (2002) observed that the ultrastructural appearance of nuclei and mitochondria did not differ in immature oocytes of RB heifers, whereas after in vitro maturation, the mitochondria were located in the periphery of the oocyte and, in RB heifers, most were abnormal in shape, in contrast to their even distribution and normal shape in virgin heifers. However, Båge et al. (2002) evaluated the oocytes only until cleavage, which was achieved by 33.0 and 36% of oocytes in RB and normal heifers, respectively, and did not continue the study until blastocyst formation; nevertheless, the mitochondrial abnormality could be significant enough to affect oocyte competence (Mermillod et al., 1999). In a recent study of oocytes collected during summer, Ferreira et al. (2016) observed less mitochondrial DNA and greater expression of mitochondrial and apoptotic genes in oocytes from RB cows than in those from heifers or peak-lactation cows. Similarly to our present findings, a study by Ferreira et al. (2011) found that

blastocyst rate, not cleavage rate, exhibited significant interactions with season (summer or winter) and group (heifer, peak lactation, or RB), along with compromised blastomere quality in RB cows. This suggests that abnormal embryo development, rather than fertilization failure, is the major cause of repeat breeding in heifers (Gustafsson, 1985).

In a previous study, Irving-Rodgers et al. (2009) found that follicles with a single-layered basal membrane had more competent oocytes than other healthy antral follicles that had a multilayered basal lamina. We recently found that expression of basement-membrane-specific heparan sulfate proteoglycan core protein (HSPG2) was lower in RB than in normal cows (Zachut et al., 2016), and Rodgers et al. (2003) found that HSPG2 affected follicular growth and its differentiation. Thus, the involvement of the basal membrane in determination of oocyte competence needs further evaluation in RB cows.

Our present study was limited by the possibility that CTL cows might shift into the RB category. However, we applied several means to exclude cows with any history, such as dystocia or retained placenta, and we monitored ovaries and uteri to minimize the likelihood of including potential RB animals in the CTL group. Also, we defined RB as cows that did not conceive after at least 4 AI, although there was a possibility that these cows might conceive as a result of the subsequent AI. In either case, the differences between CTL and RB cows could be underestimated. Another limitation is the different stage in lactation of the CTL and RB cows and the accompanying outcomes, which were derived from the study design. Nevertheless, it should be noted that the lower yields and the improved energy balance of the RB cows, as compared with their counterparts, are favorable for fertility, and even though they failed to conceive, probably due to physiological failure in the reproductive system per se. We were aware of these limitations when we planned the study but believe that they are inevitable in such a study.

In conclusion, the present study addressed 2 stages of follicular development in RB cows. Oocytes from follicles at an earlier stage of folliculogenesis yielded significantly fewer blastocysts than those from later-stage follicles, whereas FF from larger preovulatory follicles revealed an altered steroidal environment in the RB cows. These results indicate the possibility of impaired oocyte competence in early stages of development, which may impair subsequent embryo vitality and development in RB cows. Other postovulatory factors, such as uterine environment and corpus luteum functioning and vitality, might be additional causes of pregnancy failure in RB cows; these aspects were

not addressed in the present study and require further research.

ACKNOWLEDGMENTS

This research was financially supported by the Israeli Dairy Board, Yehud, Israel; 362-378-14). The authors thank the entire staff of the experimental farm of the Volcani Center for assistance with cow management.

REFERENCES

- Aardema, H., B. A. Roelen, H. T. van Tol, C. H. Oei, B. M. Gadella, and P. L. Vos. 2013. Follicular 17 β -estradiol and progesterone concentrations and degree of cumulus cell expansion as predictors of in vivo-matured oocyte developmental competence in superstimulated heifers. *Theriogenology* 80:576–583.
- Assey, R. J., P. Hyttel, J. F. Roche, and M. Boland. 1994. Oocyte steroid and follicular steroid concentrations in superovulated versus unstimulated heifers. *Mol. Reprod. Dev.* 39:8–16.
- Atkins, J. A., M. F. Smith, M. D. MacNeil, E. M. Jinks, F. M. Abreu, L. J. Alexander, and T. W. Geary. 2013. Pregnancy establishment and maintenance in cattle. *J. Anim. Sci.* 91:722–733.
- Båge, R., H. Gustafsson, B. Larsson, M. Forsberg, and H. Rodríguez-Martínez. 2002. Repeat breeding in dairy heifers: Follicular dynamics and estrous cycle characteristics in relation to sexual hormone patterns. *Theriogenology* 57:2257–2269.
- Båge, R., S. Petyim, B. Larsson, T. Hallap, A. S. Bergqvist, H. Gustafsson, and H. B. Martínez. 2003. Oocyte competence in repeat-breeder heifers: Effects of an optimized ovum pick-up schedule on expression of estrus, follicular development and fertility. *Reprod. Fertil. Dev.* 15:115–123.
- Bartlett, P. C., H. J. Kirk, and E. C. Mather. 1986. Repeated insemination in Michigan Holstein-Friesian cattle: Incidence, descriptive epidemiology and estimated economic impact. *Theriogenology* 26:309–322. [https://doi.org/10.1016/0093-691X\(86\)90150-0](https://doi.org/10.1016/0093-691X(86)90150-0).
- Ben-Rafael, Z., F. Meloni, J. F. Strauss 3rd, L. Blasco, L. Mastroianni Jr., and G. L. Flickinger. 1987. Relationships between polypronuclear fertilization and follicular fluid hormones in gonadotropin-treated women. *Fertil. Steril.* 47:284–288.
- Bloch, A., Y. Folman, M. Kaim, Z. Roth, R. Braw-Tal, and D. Wolfenson. 2006. Endocrine alterations associated with extended time interval between estrus and ovulation in high-yield dairy cows. *J. Dairy Sci.* 89:4694–4702. [https://doi.org/10.3168/jds.S0022-0302\(06\)72520-6](https://doi.org/10.3168/jds.S0022-0302(06)72520-6).
- Blondin, P., C. Vigneault, A. L. Nivet, and M. A. Sirard. 2012. Improving oocyte quality in cows and heifers—What have we learned so far? *Anim. Reprod.* 9:281–289.
- Christian, C. A., and S. M. Moenter. 2010. The neurobiology of pre-ovulatory and estradiol-induced gonadotropin-releasing hormone surges. *Endocr. Rev.* 31:544–577. <https://doi.org/10.1210/er.2009-0023>.
- Dawson, A., G. Griesinger, and K. Diedrich. 2006. Screening oocytes by polar body biopsy. *Reprod. Biomed. Online* 13:104–109.
- de Loos, F., C. van Vliet, P. van Maurik, and T. A. Kruip. 1989. Morphology of immature bovine oocytes. *Gamete Res.* 24:197–204.
- Dovensky, T., P. Trojancanec, L. J. Kosky, K. Popovsky, G. Mickovsky, V. Petkov, and L. J. Mickov. 2000. Ultrasonography of the ovaries during oestrous and the subsequent oestrous cycle in repeat breeder dairy cows. Page 18 in *Proc. 14th Int. Congr. Anim. Reprod.*, Stockholm, Sweden.
- Edmonson, A. J., I. J. Lean, L. D. Weaver, T. Fervor, and G. Webster. 1989. A body condition scoring chart for Holstein dairy cows. *J. Dairy Sci.* 89:68–78. [https://doi.org/10.3168/jds.S0022-0302\(89\)79081-0](https://doi.org/10.3168/jds.S0022-0302(89)79081-0).

- Ferreira, R. M., H. Ayres, M. R. Chiaratti, M. L. Ferraz, A. B. Araújo, C. A. Rodrigues, Y. F. Watanabe, A. A. Vireque, D. C. Joaquim, L. C. Smith, F. V. Meirelles, and P. S. Baruselli. 2011. The low fertility of repeat-breeder cows during summer heat stress is related to a low oocyte competence to develop into blastocysts. *J. Dairy Sci.* 94:2383–2392.
- Ferreira, R. M., M. R. Chiaratti, C. H. Macabelli, C. A. Rodrigues, M. L. Ferraz, Y. F. Watanabe, L. C. Smith, F. V. Meirelles, and P. S. Baruselli. 2016. The infertility of repeat-breeder cows during summer is associated with decreased mitochondrial DNA and increased expression of mitochondrial and apoptotic genes. *Biol. Reprod.* 94:66.
- Graden, A. P., D. Olds, C. R. Mochow, and L. R. Mutter. 1968. Causes of fertilization failure in repeat breeding cattle. *J. Dairy Sci.* 51:778–781.
- Gustafsson, H. 1985. Characteristics of embryos from repeat breeder and virgin heifers. *Theriogenology* 23:487–498.
- Gustafsson, H., and U. Emanuelson. 2002. Characterization of the repeat breeding syndrome in Swedish dairy cattle. *Acta Vet. Scand.* 43:115–125.
- Hawk, H. W. 1983. Sperm survival and transport in the female reproductive tract. *J. Dairy Sci.* 66:2645–2660.
- Henderson, K. M., A. S. McNeilly, and I. A. Swanston. 1982. Gonadotrophin and steroid concentrations in bovine follicular fluid and their relationship to follicle size. *J. Reprod. Fertil.* 65:467–473.
- Hyttel, P., T. Fair, H. Callesen, and T. Greve. 1997. Oocyte growth, capacitation and final maturation in cattle. *Theriogenology* 47:23–32.
- Ireland, J. J., and F. Roche. 1982. Development of antral follicles in cattle after prostaglandin-induced luteolysis: Changes in serum hormones, steroids in follicles fluids and gonadotropin receptors. *Endocrinology* 111:2077–2086.
- Irving-Rodgers, H. F., S. Morris, R. A. Collett, T. T. Peura, M. Davy, J. G. Thompson, H. D. Mason, and R. J. Rodgers. 2009. Phenotypes of the ovarian follicular basal lamina predict developmental competence of oocytes. *Hum. Reprod.* 24:936–944.
- Israel Cattle Breeders' Association. 2014. Israeli Holstein Herdbook Annual Report. Israel Cattle Breeders' Association, Ceasarea Industrial Park, Israel.
- Israel Cattle Breeders' Association. 2015. Israeli Holstein Herdbook Annual Report. Israel Cattle Breeders' Association, Ceasarea Industrial Park, Israel.
- Jimenez-Krassel, F., J. K. Folger, J. L. Ireland, G. W. Smith, X. Hou, J. S. Davis, P. Lonergan, A. C. Evans, and J. J. Ireland. 2009. Evidence that high variation in ovarian reserves of healthy young adults has a negative impact on the corpus luteum and endometrium during estrous cycles in cattle. *Biol. Reprod.* 80:1272–1281.
- Jinks, E. M., M. F. Smith, J. A. Atkins, K. G. Pohler, G. A. Perry, M. D. MacNeil, A. J. Roberts, R. C. Waterman, L. J. Alexander, and T. W. Geary. 2013. Preovulatory estradiol and the establishment and maintenance of pregnancy in suckled beef cows. *J. Anim. Sci.* 91:1176–1185.
- Kauffold, J., H. A. Amer, U. Bergfeld, W. Weber, and A. Sobiraj. 2005. The in vitro developmental competence of oocytes from juvenile calves is related to follicular diameter. *J. Reprod. Dev.* 51:325–332.
- Kreiner, D., H. C. Liu, J. Itskovitz, L. Veeck, and Z. Rosenwaks. 1987. Follicular fluid estradiol and progesterone are markers of preovulatory oocyte quality. *Fertil. Steril.* 48:991–994.
- Kruip, T. A., and S. J. Dieleman. 1985. Steroid hormone concentrations in the fluid of bovine follicles relative to size, quality and stage of the oestrus cycle. *Theriogenology* 24:395–408.
- Kurykin, J., A. Waldmann, T. Tiirats, T. Kaart, and U. Jaakma. 2011. Morphological quality of oocytes and blood plasma metabolites in repeat breeding and early lactation dairy cows. *Reprod. Domest. Anim.* 46:253–260. <https://doi.org/10.1111/j.1439-0531.2010.01652.x>.
- Li, F., X. Chen, W. Pi, C. Liu, and Z. Shi. 2007. Collection of oocytes through transvaginal ovum pick-up for in vitro embryo production in Nanyang Yellow cattle. *Reprod. Domest. Anim.* 42:666–670.
- Lonergan, P., D. Rizos, F. Ward, and M. P. Boland. 2001. Factors influencing oocyte and embryo quality in cattle. *Reprod. Nutr. Dev.* 41:427–437.
- López-Gatius, F., P. Santolaria, I. Mundet, and J. L. Yáñez. 2005. Walking activity at estrus and subsequent fertility in dairy cows. *Theriogenology* 63:1419–1429. <https://doi.org/10.1016/j.theriogenology.2004.07.007>.
- Lynch, C. O., D. A. Kenny, S. Childs, and M. G. Diskin. 2010. The relationship between periovulatory endocrine and follicular activity on corpus luteum size, function, and subsequent embryo survival. *Theriogenology* 73:190–198. <https://doi.org/10.1016/j.theriogenology.2009.08.012>.
- Mermillod, P., B. Oussaid, and Y. Cognie. 1999. Aspects of follicular and oocyte maturation that affect the developmental potential of embryos. *J. Reprod. Fertil. Suppl.* 54:449–460.
- Messinis, I. E., and A. A. Templeton. 1987. Relationship between intrafollicular levels of prolactin and sex steroids and in vitro fertilization of human oocytes. *Hum. Reprod.* 2:607–609.
- Moallem, U., Y. Folman, A. Bor, A. Arav, and D. Sklan. 1999. Effect of calcium soaps of fatty acids and administration of somatotropin on milk production, preovulatory follicular development, and plasma and follicular fluid lipid composition in high yielding dairy cows. *J. Dairy Sci.* 82:2358–2368.
- Mossa, F., F. Jimenez-Krassel, J. K. Folger, J. L. Ireland, G. W. Smith, P. Lonergan, A. C. Evans, and J. J. Ireland. 2010a. Evidence that high variation in antral follicle count during follicular waves is linked to alterations in ovarian androgen production in cattle. *Reproduction* 140:713–720.
- Mossa, F., F. Jimenez-Krassel, S. Walsh, D. P. Berry, S. T. Butler, J. Folger, G. W. Smith, J. L. Ireland, P. Lonergan, J. J. Ireland, and A. C. Evans. 2010b. Inherent capacity of the pituitary gland to produce gonadotropins is not influenced by the number of ovarian follicles ≥ 3 mm in diameter in cattle. *Reprod. Fertil. Dev.* 22:550–557.
- Mossa, F., S. W. Walsh, S. T. Butler, D. P. Berry, F. Carter, P. Lonergan, G. W. Smith, J. J. Ireland, and A. C. Evans. 2012. Low numbers of ovarian follicles ≥ 3 mm in diameter are associated with low fertility in dairy cows. *J. Dairy Sci.* 95:2355–2361. <https://doi.org/10.3168/jds.2011-4325>.
- NRC. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.
- Perez, C. C., I. Rodriguez, F. Espana, J. Dorado, M. Hidalgo, and J. Sanz. 2003. Follicular growth patterns in repeat breeder cows. *Vet. Med. Czech.* 48:1–8.
- Perez-Marin, C. C., and F. Espana. 2007. Oestrus expression and ovarian function in repeat breeder cows, monitored by ultrasonography and progesterone assay. *Reprod. Domest. Anim.* 42:449–456. <https://doi.org/10.1111/j.1439-0531.2006.00805.x>.
- Revelli, A., L. D. Piane, S. Casano, E. Molinari, M. Massobrio, and P. Rinaudo. 2009. Follicular fluid content and oocyte quality: From single biochemical markers to metabolomics. *Reprod. Biol. Endocrinol.* 7:40. <https://doi.org/10.1186/1477-7827-7-40>.
- Rodgers, R. J., H. F. Irving-Rodgers, and D. L. Russell. 2003. Extracellular matrix of the developing ovarian follicle. *Reproduction* 126:415–424.
- Roth, Z., G. Inbar, and A. Arav. 2008. Comparison of oocyte developmental competence and follicular steroid content of nulliparous heifers and cows at different stages of lactation. *Theriogenology* 69:932–939.
- SAS Institute. 2002. SAS User's Guide: Statistics. Version 9.2. SAS Institute Inc., Cary, NC.
- Saumande, J., and P. Humbolt. 2005. The variability in the interval between estrus and ovulation in cattle and its determinants. *Anim. Reprod. Sci.* 85:171–182. <https://doi.org/10.1016/j.anireprosci.2003.09.009>.
- Sood, P., M. Zachut, H. Dube, and U. Moallem. 2015. Behavioral and hormonal pattern of repeat breeder cows around estrus. *Reproduction* 149:545–554.
- Tanabe, T. Y., H. W. Hawk, and J. F. Hasler. 1985. Comparative fertility of normal and repeat-breeding cows as embryo recipients. *Theriogenology* 23:687–696.

- Tarlatzis, B. C., K. Pazaitou, H. Bili, J. Bontis, J. Papadimas, S. Lagos, E. Spanos, and S. Mantalenakis. 1993. Growth hormone, oestradiol, progesterone and testosterone concentrations in follicular fluid after ovarian stimulation with various regimes for assisted reproduction. *Hum. Reprod.* 8:1612–1616.
- Teissier, M. P., H. Chable, S. Paulhac, and Y. Aubard. 2000. Comparison of follicle steroidogenesis from normal and polycystic ovaries in women undergoing IVF: relationship between steroid concentrations, follicle size, oocyte quality and fecundability. *Hum. Reprod.* 15:2471–2477.
- Tesarik, J., and C. Mendoza. 1997. Direct non-genomic effects of follicular steroids on maturing human oocytes: oestrogen versus androgen antagonism. *Hum. Reprod. Update* 3:95–100.
- Uehara, S., T. Naganuma, A. Tsuiki, K. Kyono, H. Hoshiai, and M. Suzuki. 1985. Relationship between follicular fluid steroid concentrations and in vitro fertilization. *Obstet. Gynecol.* 66:19–23.
- Van der Schans, A., L. A. J. Van der Westerlaken, A. A. C. De Wit, W. H. Eyestone, and H. A. De Boer. 1991. Ultrasound-guided transvaginal collection of oocytes in the cow. *Theriogenology* 35:288. (Abstr.)
- Vanderhyden, B. C., J. N. Cohen, and P. Morley. 1993. Mouse oocytes regulate granulosa cell steroidogenesis. *Endocrinology* 133:423–426. <https://doi.org/10.1210/en.133.1.423>.
- Walters, K. A., C. M. Allan, and D. J. Handelsman. 2008. Androgen actions and the ovary. *Biol. Reprod.* 78:380–389.
- Webb, R., and B. K. Campbell. 2007. Development of the dominant follicle: Mechanisms of selection and maintenance of oocyte quality. *Soc. Reprod. Fertil. Suppl.* 64:141–163.
- Wehrman, M. E., M. S. Roberson, A. S. Cupp, F. N. Kojima, T. T. Stumpf, L. A. Werth, M. W. Wolfe, R. J. Kittok, and J. F. Kinder. 1993. Increasing exogenous progesterone during synchronization of estrus decreases endogenous 17 β -estradiol and increases conception in cows. *Biol. Reprod.* 49:214–220.
- Yusuf, M., T. Nakao, R. B. Ranasinghe, G. Gautam, S. T. Long, C. Yoshida, K. Koike, and A. Hayashi. 2010. Reproductive performance of repeat breeders in dairy herds. *Theriogenology* 73:1220–1229.
- Zachut, M., I. Dekel, H. Lehrer, A. Arieli, A. Arav, L. Livshitz, S. Yakoby, and U. Moallem. 2010. Effects of dietary fats differing in n-6:n-3 ratio fed to high-yielding dairy cows on fatty acid composition of ovarian compartments, follicular status, and oocyte quality. *J. Dairy Sci.* 93:529–545.
- Zachut, M., P. Sood, Y. Levin, and U. Moallem. 2016. Proteomic analysis of pre-ovulatory follicular fluid reveals differentially abundant proteins in less-fertile dairy cows. *J. Proteomics* 139:122–129.