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To cite this article: Angela Salzano, Bianca Gasparri, Domenico Vecchio, Valentina Longobardi, Pietro Sampaio Baruselli, Anna Balestrieri, Francesca Licitra, Michael D'Occhio & Gianluca Neglia (2019) Effect of photoperiod on follicular IGF-1 and oocyte quality independently of metabolic status in buffalo heifers, Italian Journal of Animal Science, 18:1, 949-956, DOI: [10.1080/1828051X.2019.1588793](https://doi.org/10.1080/1828051X.2019.1588793)

To link to this article: <https://doi.org/10.1080/1828051X.2019.1588793>



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Published online: 22 May 2019.



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## Effect of photoperiod on follicular IGF-1 and oocyte quality independently of metabolic status in buffalo heifers

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### ABSTRACT

The aim of this study was to determine whether buffalo heifers maintained at a constant live weight (LW) and body condition score (BCS) by a restricted diet show changes in reproductive function in response to changes in day length. Heifers were fed a low energy diet (86% daily intake recommended for the species) throughout the study, which involved an 8-week period of decreasing day length and a 7-week period of increasing day length. Weekly, LW, BCS and ovarian follicular/oocyte population were assessed. Blood and follicular fluid samples were taken at the time of ovum pick-up for hormone analyses and at 30-day intervals to measure metabolic substrates. Buffalo heifers maintained LW and BCS throughout the study and blood concentrations of the main metabolic hormones, such as GH, insulin, IGF-1 and thyroid hormones were not influenced by photoperiod. Likewise, there were no differences in metabolic substrates such as glucose, NEFA, triglycerides and HDL cholesterol, suggesting that the metabolic condition remained essentially the same. During increasing day length periods, the number of total follicles ( $8.4 \pm 0.5$  vs.  $11.9 \pm 0.6$ ;  $p < .01$ ), cumulus-oocyte complexes ( $2.2 \pm 0.3$  vs.  $4.3 \pm 0.5$ ;  $p < .01$ ) and superior quality Grade A+B COCs ( $0.2 \pm 0.1$  vs.  $1.1 \pm 0.2$ ;  $p < .01$ ) decreased. These features were associated to reduced oestradiol levels both in plasma ( $3.9 \pm 0.4$  vs.  $9.2 \pm 0.5$  pg/mL;  $p < .01$ ) and follicular fluid ( $49.7 \pm 12.9$  vs.  $109.1 \pm 25.8$  ng/mL;  $p = .05$ ), as well as to reduced intrafollicular IGF-1 levels ( $45.5 \pm 4.0$  vs.  $58.5 \pm 3.9$  ng/mL;  $p < .05$ ). These findings suggest that the ovarian function in buffalo heifers is influenced by photoperiod independent of nutritional and metabolic status.

### HIGHLIGHTS

- Metabolic profile, LW and BCS do not change in buffalo heifers on a restricted diet
- Photoperiod affects ovarian function in heifers regardless of metabolic status
- Follicular and oocyte population decrease in increasing day length periods
- Oestradiol and IGF-1 levels in follicular fluid are affected by photoperiod

### ARTICLE HISTORY

Received 25 October 2018  
Revised 22 January 2019  
Accepted 24 February 2019

### KEYWORDS

Photoperiod; oocyte quality; metabolic status; buffalo heifers

## Introduction

River buffalo (*Bubalus bubalis*) is important global livestock for milk and meat. Buffaloes have a relatively high efficiency of feed conversion and maintain productivity on diets that are restrictive for cattle (Campanile et al. 2010c). A feature of this species is the ability to adapt to different environments ranging from the equator to higher latitudes (Vale 2007). Buffaloes are reproductively photoperiodic and in sub-

tropical and temperate regions show a seasonal increase in fertility in response to decreasing day length (Baruselli 1993; Zicarelli 1997). Indeed, improved oocyte competence (Di Francesco et al. 2011; Di Francesco, Neglia et al. 2012), greater secretion of progesterone ( $P_4$ ) by the CL (Di Francesco, Novoa et al. 2012), lower incidence of embryonic mortality (Campanile et al. 2005; Campanile and Neglia 2007; Campanile, Baruselli, Neglia et al. 2010), as well

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as shorter periods of postpartum anoestrus (Gupta et al. 2015) are observed during decreasing day length months. The pattern of seasonality linked to photoperiod is more evident at higher latitudes (Zicarelli 1997; Campanile, Baruselli, Neglia et al. 2010) while in equatorial zones nutrition has an important role due to fluctuations in forage quantity and quality. Nevertheless, in all environments an increase in fertility depends on the metabolic and reproductive endocrine status, resulting in optimal follicular, oocyte and embryonic development, appropriate CL function, ensuring the establishment of pregnancy.

In an earlier study, metabolic status and reproductive function were monitored in Murrah buffalo heifers fed high energy (HE) or low energy (LE) diet (Campanile, Baruselli, Vecchio et al. 2010). Heifers on HE diet had higher plasma concentrations of insulin, leptin and T3, as well as greater follicular fluid concentrations of IGF-1 and higher oocyte quality index, despite similar ovarian follicular characteristics. It is known that IGF-1 plays important roles in growth and function of the granulosa and thecal layers (Chouhan et al. 2014, 2015; Uniyal et al. 2015), oocyte development (Totey et al. 1996; Pawshe et al. 1998), steroidogenesis (Babitha et al. 2014; Chouhan et al. 2014; Singh et al. 2015) and vascular endothelial growth factor (VEGF) production by granulosa and thecal cells (Babitha et al. 2014; Chouhan et al. 2015). Murrah heifers on LE diet (86% recommended requirements according to Campanile et al. 2001; NRC 2001) maintained relatively constant live weight (LW) and body condition score (BCS) across periods of decreasing and increasing day length (Campanile, Baruselli, Vecchio et al. 2010b). In the sheep (Ile-de-France and Spanish Rasa Aragonesa breeds) reproductive function was demonstrated to vary in response to photoperiod, independently of changes in LW and BCS (Forcada et al. 1992; Forcada and Abecia 2006; Menassol et al. 2012). The two studies in ewes showed that whilst nutrition and photoperiod are usually coupled to influence reproduction (Fernandez-Fernandez et al. 2006; Scaramuzzi and Martin 2008), reproductive responses to photoperiod can occur in ewes without changes in metabolic status. It is not possible to rule out that this is a general phenomenon across small and large ruminants. Therefore, the aim of the present study was to determine whether buffalo heifers maintained at a constant LW and BCS showed changes in reproductive function in response to changes in day length. Reproductive function was ascertained by measuring plasma and follicular IGF-1, steroidogenesis, folliculogenesis and oocyte quality.

## Materials and methods

The present study analysed information obtained as part of an earlier report (Campanile, Baruselli, Vecchio et al. 2010). The Ethical Animal Care and Use Committee of the Federico II University of Naples approved the experimental design and animal treatments.

### Animals and management

Six Murrah River buffalo heifers (age,  $781 \pm 22$  days; LW,  $404 \pm 12$  kg; BCS,  $3.7 \pm 0.1$ ) had been transferred to the São Paulo University (São Paulo, Brazil), where the study was carried out, from natural sub-tropical pastures. After the transfer animals were allowed to acclimatise to the housing and diet for 7 weeks before sampling commenced. Heifers were maintained in open yards ( $20 \text{ m}^2/\text{animal}$ ) and fed 6 kg of *Brachiaria Hay* and 0.2 kg of vitamin-mineral supplementation daily for the entire duration of the study. The diet was characterised by 3.6 milk forage units (MFU) which corresponded to 86% of the recommended requirements (Campanile et al. 2001; NRC 2001).

### Sampling design

The six buffalo heifers were maintained under the same above described management throughout the study which involved an eight-week period of decreasing day length (autumn, April–June) and a seven-week period of increasing day length (spring, October–November). LW and BCS (scale 1–5; Baruselli et al. 2001) were recorded weekly by the same operator. Ovarian follicular status and oocyte quality were assessed weekly in conjunction with ovum pick-up (OPU) (Neglia et al. 2003, 2011). Blood samples were taken weekly (at the time of OPU) for hormone analyses and at 30-day intervals to measure metabolic substrates. All blood samples were centrifuged at  $800 \times g$  for 15 min and serum or plasma was stored at  $-18^\circ\text{C}$  until required for analysis. Likewise, the follicular fluid of the dominant follicle was collected at the time of OPU for hormone analyses and at 30-day intervals to measure metabolic substrates.

### OPU, ovarian follicular population and oocyte classification

The size of the ovaries was assessed by measuring the two diameters using an ultrasonography unit (SonoAce – PICO, Medison, Cypress, CA) with a convex 4 MHz - 9 MHz probe (mod. CD49/10EDN). Ovum pick-up involved the ultrasonography unit above described

(that was equipped with a metal guide for 18-gauge aspiration needles (WTA Ltda., Cravinhos/SP, Brazil). A vacuum pressure of 40 mm Hg was constantly maintained by using a suction unit (K-MAR-5100, Cook IVF Co., Australia) and the aspiration line was continuously rinsed with 25 mM hepes buffered TCM 199 supplemented with 100 USP units mL<sup>-1</sup> of heparin, 10% foetal calf serum (FCS) and 1% penicillin and streptomycin complex (20,000 U and 20,000 µg/mL, respectively, Lonza, Milan, Italy) during follicular aspiration. Oocytes were collected into 15 mL Falcon tubes (Becton & Dickinson Co., Lincoln Park, NJ) that were constantly maintained at 37 °C.

The follicular fluid of the largest follicle (around 13 mm diameter) was collected separately and all other follicles visible by ultrasound were aspirated to recover cumulus oocyte complexes (COCs). The follicles were counted and classified as: small, < 5 mm diameter; medium, 5 – 10 mm diameter; large, >10 mm diameter. Moreover, the number of oocytes recovered was recorded. COCs were isolated immediately after OPU by filtering the aspirated follicular fluid and aspiration medium and classified into five categories: Grade A, oocytes with more than three layers of granulosa cells and homogenous cytoplasm; Grade B, oocytes with at least two layers of granulosa cells and homogenous cytoplasm; Grade C, partially or totally denuded; Grade D, degenerated oocytes; Grade E, expanded oocytes but with homogeneous cytoplasm, typical of the oestrous phase. The number and percentage of good quality COCs (Grade A + B) and COCs considered suitable for IVF (Grade A + B + C) were recorded.

### **Plasmatic metabolic substrates**

Total cholesterol, HDL cholesterol, glucose, triglycerides and non-esterified fatty acids (NEFA) were measured by an enzymatic colorimetric method commercially available as previously described (Campanile et al. 2003). Respective intra- and inter-assay coefficients of variation were 2.0% and 3.2% (total cholesterol), 1.1% and 2.0% (HDL cholesterol), 1.8% and 2.0% (triglycerides) and 2.5% and 3.8% (NEFA). Assay sensitivities were 0.6 mg (total cholesterol), 0.4 mg (HDL cholesterol), 1.6 mg (triglycerides) and 0.02 mM (NEFA).

### **Metabolic hormones**

Insulin was measured in plasma by the commercially available automated fluoro-2-immunometric method as previously described (Campanile et al. 2003). Intra- and inter-assay coefficients of variation were 9.6% and

6.4%, respectively, and the assay sensitivity was 0.7 mM. Glucagon was measured in plasma by a radioimmunoassay kit (LINCO Research Inc., St. Charles, MO). Intra- and inter-assay coefficients of variation were 9.5% and 9.9%, respectively, and the assay sensitivity was 3.0 pM. Plasma concentrations of leptin were determined using a multispecies radioimmunoassay kit (LINCO Research Inc., St. Charles, MO). Serial dilutions of buffalo plasma had parallelism with the leptin standard curve. The sensitivity of the assay was 0.37 ng and the intra- and inter-assay coefficients of variation were 4.2 and 8.0%, respectively.

Concentrations of IGF-1 were determined in plasma and follicular fluid by double antibody radioimmunoassay (RIA) (Lemal et al. 1989; Renaville et al. 1993). In this method, a cryoprecipitation step is used to remove aggregated IGF-1 binding proteins. After acid-ethanol extraction (87.5% ethanol and 12.5% 2M HCL) an aliquot of the supernatant was neutralised with 0.855M Tris base at a ratio of 5:2. The samples were then stored at –20 °C for 1 h and then centrifuged at 3000 rpm for 30 min at 4 °C. The supernatant was decanted into fresh tubes and used in the RIA. The recovery of IGFI added to plasma was 94.4 ± 3.2%. Recombinant human IGFI (Roche, Mannheim, Germany) was used as the standard and for preparing radiolabelled IGF-1 (Salacinski et al. 1981). Aliquots of 6000 cpm <sup>125</sup>I-IGFI (specific activity: 130.7 µCi/µg) were added to assay tubes. The primary antibody was rabbit anti-human IGF-1 diluted at 1:30,000 (GroPep, Adelaide, Australia) and secondary antibody was goat anti-rabbit IgG (GroPep, Adelaide, Australia). The minimum detectable amount of IGFI was 1.3 ng and the intra- and inter-assay coefficients of variation were 8 and 12%, respectively.

Circulating concentrations of GH were determined using a heterologous double antibody RIA (Salacinski et al. 1981). The assay materials were (1) rabbit anti-bovine GH antiserum as primary antibody diluted at 1:1750, (2) ovine GH (LER 1774) used to prepare both <sup>125</sup>I GH (Salacinski et al. 1981) and graded standards to generate standard displacement curves and (3) goat anti-rabbit IgG as secondary antibody. The minimum detectable amount of GH was 0.4 ng and the intra- and inter-assay coefficients of variation were 6.9 and 11.9%, respectively.

Serum total triiodothyronine (T3) was determined by radioimmunoassay (Chopra, Solomon, Ho 1971). Duplicate samples of 100 µL were assayed together with T3 standards ranging from 25 to 800 ng/dL. Assay sensitivity was 4.5 ng and the intra and inter-assay coefficients of variance were 2.5% and 6.0%, respectively.

Serum concentrations of total thyroxine (T4) were determined by radioimmunoassay (Chopra, Solomon, Beall 1971). Duplicate samples of 20  $\mu$ L were assayed together with T4 standards ranging from 2 to 32  $\mu$ g/dL. Assay sensitivity was 2.5  $\mu$ g and the intra- and inter-assay coefficients of variation were 3.6 and 8.2%, respectively. Concentrations of thyroid-stimulating hormone (TSH) were determined in a single assay (Borger and Davis 1974). Assay sensitivity was 3.0 ng and the intra-assay coefficient of variation was 1.3%.

### Oestradiol and progesterone

Oestradiol and progesterone were determined in plasma and follicular fluid by radioimmunoassay (Seren et al. 1974). The oestradiol antiserum was rabbit anti-oestradiol 6-(0-carboxymethyl) oxime-BSA and was used at a final dilution of 1:31,500. The minimum detectable amount of oestradiol was 0.82 pg and the intra- and inter-assay coefficients of variation were 8.6% and 12.5%, respectively. The progesterone antiserum was rabbit anti-17 $\alpha$ -hydroxyprogesterone-3 (O-carboxymethyl) oxime-BSA and was used at a final dilution of 1:6000. The minimum detectable amount of progesterone was 2.1 pg and the intra- and inter-assay coefficients of variation were 6.2 and 11.8%, respectively.

### Statistical analyses

Differences between periods of increasing and decreasing daily length in metabolic substrates and hormones, as well as in reproductive parameters were analysed by Student's *T*-test. In case of non-homogeneity of variance, the non-parametric Mann-Whitney test was used. Multiple linear regression was used to evaluate factors affecting the number of follicles and COCs, and also the number and percentage of good quality COCs (Grade A+B), both independently of photoperiod and within each photoperiod.

## Results

### Live weight and body condition

The LW of heifers did not statistically differ between the periods of decreasing (411  $\pm$  4 kg) and increasing (406  $\pm$  3 kg) day length. The BCS also did not differ between decreasing (3.56  $\pm$  0.03) and increasing (3.53  $\pm$  0.04) day length.

### Metabolic substrates

Results for the concentrations of metabolic substrates are shown in Table 1. Blood concentrations of glucose, NEFA, triglycerides and HDL cholesterol did not differ between decreasing and increasing day length. Total cholesterol was greater ( $p < .01$ ) during the period of decreasing day length.

### Metabolic hormones

Results for the concentrations of metabolic hormones are shown in Table 2. Blood concentrations of insulin, IGF-1, GH, T3, T4 and TSH did not differ between the periods of decreasing and increasing day length. Blood concentrations of glucagon were lower ( $p < .05$ ) and concentrations of leptin were higher ( $p < .01$ ) during decreasing day length compared with increasing day length. Despite the similar concentrations in blood, intra-follicular IGF-1 was higher ( $p < .05$ ) during decreasing day length (Table 3).

### Oestradiol and progesterone

Concentrations of oestradiol were greater during the period of decreasing day length in both blood ( $p < .01$ ) and follicular fluid ( $p = .05$ ), as shown in Table 3. Concentrations of P<sub>4</sub> in blood and follicular fluid did not differ between decreasing and increasing day length (Table 3).

**Table 1.** Blood concentrations (mean  $\pm$  SEM) of metabolic substrates in buffalo heifers ( $n = 6$ ) during periods of decreasing and increasing day length.

	Day length		<i>p</i> value
	Decreasing	Increasing	
Total cholesterol, mg/dL	69.0 $\pm$ 2.1	58.0 $\pm$ 2.9	.01
HDL cholesterol, mg/dL	44.3 $\pm$ 1.8	50.0 $\pm$ 5.1	.20
Glucose, mg/mL	81.5 $\pm$ 2.4	73.8 $\pm$ 1.7	.07
Tryglicerides, mg/dL	19.6 $\pm$ 2.3	18.0 $\pm$ 3.3	.73
NEFA, mmol/L	0.4 $\pm$ 0.0	0.6 $\pm$ 0.1	.07

HDL: high-density lipoprotein; NEFA: non-esterified fatty acids

**Table 2.** Blood concentrations (mean  $\pm$  SEM) of metabolic hormones in buffalo heifers ( $n = 6$ ) during periods of decreasing and increasing day length.

	Day length		<i>p</i> value
	Decreasing	Increasing	
Insulin, mmol/L	4.0 $\pm$ 0.3	3.5 $\pm$ 0.3	.41
Glucagon, pg/mL	358.5 $\pm$ 19.0	432.8 $\pm$ 19.0	.04
Leptin, ng/mL	2.1 $\pm$ 0.1	1.6 $\pm$ 0.1	<.01
IGF-1, ng/mL	84.7 $\pm$ 4.5	78.5 $\pm$ 3.4	.27
GH, pg/mL	5.9 $\pm$ 0.6	4.5 $\pm$ 1.2	.28
T3, ng/mL	163.3 $\pm$ 11.2	187.0 $\pm$ 42.0	.46
T4, $\mu$ g/mL	4.6 $\pm$ 0.3	4.7 $\pm$ 0.5	.77
TSH, $\mu$ g/mL	0.2 $\pm$ 0.1	0.3 $\pm$ 0.2	.64

IGF-1: Insulin-like growth factor 1; GH: Growth hormone; T3: triiodothyronine; T4: thyroxine; TSH: Thyroid-stimulating hormone

**Table 3.** Hormone concentrations (mean  $\pm$  SEM) in plasma and follicular fluid of buffalo heifers ( $n=6$ ) during periods of decreasing and increasing day length.

	Day length		<i>p</i> value
	Decreasing	Increasing	
Plasma			
Oestradiol, pg/mL	9.2 $\pm$ 0.5	3.9 $\pm$ 0.4	<.001
P <sub>4</sub> , ng/mL	1.9 $\pm$ 0.2	2.2 $\pm$ 0.3	.470
Follicular fluid			
Oestradiol, ng/mL	109.1 $\pm$ 25.8	49.7 $\pm$ 12.9	.050
P <sub>4</sub> , ng/mL	123.5 $\pm$ 27.9	68.3 $\pm$ 15.5	.100
IGF-1, ng/mL	58.5 $\pm$ 3.9	45.5 $\pm$ 4.0	.020

P4: Progesterone; IGF-1: Insulin-like growth factor 1

### Follicular population and oocyte quality

The size of the ovaries did not differ between the periods of decreasing and increasing day length (Table 4). The number of total follicles and small follicles was lower ( $p < .01$ ) during increasing day length whilst that of medium follicles was lower ( $p < .01$ ) during increasing day length (Table 4). The volume of the largest follicle was lower ( $p < .05$ ) in increasing day length.

There was no difference in the recovery rate of COCs between decreasing and increasing day length (Table 4). During decreasing day length there was an increase in the number of total COCs recovered ( $p < .01$ ), as well as Grade A ( $p < .05$ ), Grade B ( $p < .01$ ), Grade C ( $p < .01$ ) and Grade E ( $p < .05$ ) COCs, as shown in Table 4. The number of good quality COCs (Grade A+B) and COCs deemed suitable for IVF (Grade A+B+C) were greater ( $p < .01$ ) during decreasing day length. The number ( $p < .05$ ) and incidence ( $p < .01$ ) of Grade D COCs were greater during increasing day length (Table 4).

### Multiple linear regressions

Although no effects were observed independently from the season and in the period with decreasing day length, the total number of Grade A+B COCs were positively influenced ( $R^2 = 0.555$ ,  $p < .05$ ) by plasma GH concentrations during the period of increasing day length according to the equation:

$$\text{COCs Grade A + B} = 0.514 + (0.0184 * \text{plasma GH})$$

The percentage of Grade A+B COCs was also positively influenced ( $R^2 = 0.66$ ,  $p < .05$ ) by plasma GH concentrations during the period of increasing day length according to the equation:

$$\% \text{ COCs Grade A + B} = 38.106 + (1.863 * \text{plasma GH})$$

### Discussion

The present work was designed to evaluate the effect of daily light hours on seasonal ovarian activity in

**Table 4.** Ovarian features (mean  $\pm$  SEM) of buffalo heifers ( $n=6$ ) during periods of decreasing and increasing day length.

	Day length		<i>p</i> value
	Decreasing	Increasing	
Ovarian size, cm <sup>2</sup>	3.4 $\pm$ 0.1	3.3 $\pm$ 0.1	.600
Follicular fluid, mL	1.5 $\pm$ 0.1	1.4 $\pm$ 0.0	.010
Total follicles, <i>n</i>	11.9 $\pm$ 0.6	8.4 $\pm$ 0.5	<.001
Small follicles, <i>n</i>	10.8 $\pm$ 0.6	5.3 $\pm$ 0.5	<.001
Medium follicles, <i>n</i>	1.0 $\pm$ 0.3	2.7 $\pm$ 0.3	<.001
Large follicles, <i>n</i>	0.2 $\pm$ 0.1	0.3 $\pm$ 0.1	.120
Total COCs, <i>n</i>	4.3 $\pm$ 0.5	2.2 $\pm$ 0.3	<.001
Recovery rate, %	36.2 $\pm$ 4.0	31.5 $\pm$ 6.6	.530
Grade A COCs ( <i>n</i> ), %	0.2 $\pm$ 0.1 (4.1 $\pm$ 1.6)	0.02 $\pm$ 0.0 (1.5 $\pm$ 1.5)	.040 .352
Grade B COCs ( <i>n</i> ), %	0.9 $\pm$ 0.2 (21.4 $\pm$ 4.0)	0.2 $\pm$ 0.1 (12.7 $\pm$ 4.8)	.004 .044
Grade C COCs ( <i>n</i> ), %	2.1 $\pm$ 0.3 (49.6 $\pm$ 4.9)	0.6 $\pm$ 0.2 (26.1 $\pm$ 5.8)	<.001 .003
Grade D COCs ( <i>n</i> ), %	0.5 $\pm$ 0.2 (12.4 $\pm$ 3.7)	1.1 $\pm$ 0.2 (52.1 $\pm$ 6.8)	.008 <.001
Grade E COCs ( <i>n</i> ), %	0.5 $\pm$ 0.1 (12.6 $\pm$ 3.5)	0.2 $\pm$ 0.1 (7.6 $\pm$ 2.9)	.182 .345
Grade A + B ( <i>n</i> ), %	1.1 $\pm$ 0.2 (25.5 $\pm$ 4.2)	0.2 $\pm$ 0.1 (14.2 $\pm$ 4.9)	.002 .030
Grade A + B + C ( <i>n</i> ), %	3.2 $\pm$ 0.4 (75.1 $\pm$ 5.1)	0.9 $\pm$ 0.2 (40.3 $\pm$ 6.8)	<.001 < .001

COCs: Cumulus oocyte complexes

buffalo heifers maintained at constant LW and BCS levels. This model provides a clear picture of photoperiodic effects, by ruling out nutrition-dependent seasonal changes. It was demonstrated that during increasing day length periods the follicular population and the oocyte quality decreased and these features were related to reduced plasmatic and intrafollicular oestradiol levels and intrafollicular IGF-1 concentrations.

Buffalo heifers fed a low energy diet maintained LW and BCS throughout the study. In agreement with this pattern, blood concentrations of the main hormones associated with the metabolic state, such as GH, insulin, IGF-1 and thyroid hormones were not influenced by the daily light hours. Likewise, there were no differences in metabolic substrates such as glucose, NEFA, triglycerides and HDL cholesterol. Differences were only recorded in leptin levels and total cholesterol that decreased and in glucagon levels that increased during long day months. The higher glucagon concentration during increasing day length period was unexpected given similar circulating levels of glucose, NEFA and tryglycerides in the two seasons. The decrease of plasmatic leptin during increasing day length period is also difficult to interpret as heifers did not lose any weight and maintained the same nutritional status during the trial. In sheep, circulating leptin and leptin gene expression are modulated by day length, independently of food intake and nutritional status, with lower levels associated with short days (Bocquier et al. 1998). An effect of photoperiod on

plasma leptin concentration was also observed in our study but with an opposite pattern, as lower values were recorded during long days months even if this could be also due to the longer term of LE diet to which buffalo heifers were maintained. However, taken together all these findings it can be concluded that the metabolic condition of the heifers remained essentially the same during the study.

In this study, the ovarian status of heifers kept at constant LW and BCS was affected by the photoperiod at different levels. We focussed on the ovarian activity, assessed by measuring IGF-1 in plasma and follicular fluid, the steroidogenesis, the folliculogenesis and the oocyte quality.

A first result is that season influenced the follicular population, with a decreased number of total follicles recorded in the increasing day length period, mainly due to the lower number of small follicles. This resulted in a decreased number of COCs and good quality COCs. A reduction in the number of follicles and oocytes was also recorded in Indian River buffaloes during the non-breeding season (Manjunatha et al. 2008). However, these findings are in contrast with a previous study carried out in Italian Mediterranean buffalo cows that undergone OPU in different seasons, where both the follicular population and the oocyte quality, assessed by morphological criteria, were not affected by season (Di Francesco, Neglia et al. 2012b). Nevertheless, a seasonal effect was observed on oocyte developmental competence, markedly increased during short day months, as indicated by higher blastocyst yields (Di Francesco, Neglia et al. 2012). An improved oocyte competence, despite a similar number of total COCs and good quality COCs, in short day months was also observed in a long-term retrospective study performed on abattoir-derived ovaries from Italian Mediterranean buffalo (Di Francesco et al. 2011). It is not possible to rule out that the reduced follicular and oocyte population recorded in the increasing day length period in the present study could be related to the higher number of OPU sessions heifers underwent. Indeed, it was previously demonstrated in a long-term OPU trial that repeated follicular aspiration results in a decrease of the number of follicles and COCs, likely due to scar formation in the ovarian cortex and stroma, reducing oocyte recovery efficiency overtime (Neglia et al. 2011). However, the proportion of good quality oocytes was not affected in buffaloes undergoing multiple OPU sessions overtime (Neglia et al. 2011). In the present study in Murrah buffalo heifers in addition to the decreased proportion of Grade A + B + C COCs, i.e. those considered suitable for IVF, a higher incidence

of degenerated oocytes was recorded in the increasing day length period, in contrast to previous reports (Di Francesco et al. 2011, 2012; Neglia et al. 2011). These small discrepancies among studies may be due to differences in breed, management and donors' age (heifers vs cows). A common denominator of these works is, however, the evidence of a seasonal effect on ovarian activity.

The worse oocyte quality in the long day period was accompanied by decreased oestradiol levels both in plasma and in the follicular fluid and, even more interestingly, by lower intrafollicular levels in IGF-1. It is known that IGF-1 plays a key role in ovarian follicular development, acting synergistically with FSH to increase the proliferation and oestradiol production of granulosa cells (Gong et al. 1994; Glister et al. 2001). In our study, the decreased intrafollicular concentration of IGF-1 recorded during increasing day length months was associated with reduced oestradiol synthesis, whereas progesterone levels remained similar both in plasma and in the follicular fluid in the two periods considered. It is likely that the reduced oestradiol concentration is due to the reduced intrafollicular IGF-1, although, as FSH and LH were not measured, it is not possible to rule out that it also depends on decreased gonadotrophins levels. It is worth noting that the intrafollicular levels of IGF-1 decreased in the increasing daily light hours period, while plasmatic concentrations of IGF-1, insulin and GH were similar. The reduced follicular levels in the unfavourable season may be caused by either reduced sequestration of IGF-1 from blood, decreased local production or reduced bioavailability. The latter is regulated by IGF-BPs (Armstrong and Webb 1997), that in turn are modulated by serine metalloproteases, including pregnancy-associated plasma protein-A (Mazerbourg et al. 2000). The data available do not allow to characterise the mechanism by which follicular IGF-1 decreases during the non-breeding season in buffalo heifers, suggesting further investigations in future studies. However, it is evident that a deterioration of oocyte quality was linked to decreased intrafollicular IGF-1 and decreased oestradiol production. This could be due to the known role of IGF-1 in supporting oocyte viability (Walters et al. 2006) and maturation (Izadyar et al. 1997). It is also known that IGF-1 stimulates follicular VEGF production in ruminants (Schams et al. 2001). It was reported that the amount of VEGF in the developing CL of buffalo was greater during decreasing day length and this was associated with greater angiogenesis, increased P<sub>4</sub> secretion and increased the likelihood of pregnancy (Campanile et al. 2010c;

Neglia et al. 2015). The findings in the present study could be interpreted to suggest that increased follicular IGF-1 during decreasing day length results in follicles that are more highly vascularised through VEGF, and this allows follicles to transition to a well vascularised and functional CL (Shimizu 2016).

## Conclusions

In conclusion, the ovarian function in buffalo heifers is influenced by photoperiod independent of nutritional and metabolic status, as indicated by the decreased follicular population and worse oocyte quality, associated to reduced oestradiol production and follicular IGF-1 during spring in animals maintained at constant LW and BCS. Taken together, the studies in sheep and buffalo could be interpreted to suggest that changes in reproduction that are independent of the metabolic condition are a shared phenomenon in ruminants.

## Ethical approval

The Ethical Animal Care and Use Committee of the Federico II University of Naples approved the experimental design and animal treatments.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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