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PAPER

Feedlot rearing versus pasture grazing enhances plasma leptin and insulin-like growth factor-1 concentrations but does not anticipate puberty in dairy buffalo (*Bubalus bubalis*) heifers

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ABSTRACT

Plasma Leptin (LEP) and Insulin-like Growth Factor-1 (IGF-1), related to growth and puberty onset, were assayed in growing buffalo heifers from 8 up to 21 months of age, reared in feedlot (FR, $n = 13$; initial body weight 132 ± 11 Kg) or grazing at pasture (PG, $n = 13$; 137 ± 12 Kg). The mean age at puberty was not different between the two groups (599.5 and 610.5 days, in FR and PG, respectively). At puberty, FR heifers showed higher BCS, and higher average daily weight gain (DWG) than PG ones (0.87 vs. 0.62 Kg/day), and were heavier (462 vs. 375 Kg). A negative correlation between DWG and age at puberty was significant for the PG group. Plasma Inhibin-A increased in both groups one month before puberty. Plasma LEP and IGF-1 sharply increased 2 months before puberty (about 18 months old) in FR heifers only, which showed higher concentrations of both hormones than PG heifers. Plasma LEP levels correlated positively with body weight and IGF-1. Despite hormone levels being affected by rearing systems, and being lower in heifers grazing at pasture, these latter experienced adequate growth and reproductive maturation. In fact, grazing heifers reached puberty at the same age as the feedlot reared ones, with shorter puberty–conception interval (51.3 vs. 67.2 days, for PG and FR groups, respectively) and no difference in conception rates (83.3 and 84.6%, for PG and FR groups respectively). The higher BW and DWG of FR heifers were due to body fat deposition, which did not anticipate the puberty onset.

HIGHLIGHTS

- Metabolic hormones Leptin and IGF-1, linked to puberty attainment, are good indicators of the animal energetic state.
- Buffalo heifers reared in intensive system vs. the ones at pasture had higher hormone levels and growth, but reproductive performance was not better.
- Post-weaned heifers can be reared at pasture during growth and breeding, without compromising reproductive performance.

Introduction

Water buffalo (*Bubalus bubalis*) is a prominent livestock species for developing countries in tropical and sub-tropical environments, in low-input and low-cost production systems, characterised by relatively low maintenance requirements and higher feed conversion efficiency compared with that cattle (Paul et al. 2002). In Italy, buffalo farming has been traditionally conducted for centuries by extensive rearing systems. During the last decades, buffalo rearing has been subjected to a marked intensification of breeding techniques, shifting to commercial dairy farms and large business enterprises. The European Union policy points out the need to de-intensify animal production, with attention focussed on sustainable development of marginal Mediterranean areas, often unsuitable for other production practices. An extensive and pasture-based rearing system could be a management option for raising dairy heifers: it can provide high-quality forage, can potentially lower the cost of production, allows the reduction of several sources of pollution, can reduce the competition with humans in terms of food, can increase animal welfare and is perceived by consumers as ethically sound (Sabia et al. 2018).

On the other hand, in young animals, insufficient feed intake and/or an unbalanced ration may reduce growth performances and delay the onset of puberty (Foster 1994), also in buffaloes (Pasha 2013), with a consequent reduction of the efficiency of the farms. In recent years, the relationships of nutrition with neuro-endocrine mechanisms related to the onset of puberty have been described in bovine species (Perry 2016; Cardoso et al. 2018).

Leptin (LEP) is a protein hormone secreted primarily by adipose tissue, with major endocrine effects to regulate food intake and energy expenditure (Friedman and Halaas 1998). Body weight gains and adipose tissue accretion during juvenile development can enhance the synthesis and release of LEP (Garcia et al. 2002), which signals to the central nervous system the availability of enough nutritional reserves to support the pubertal transition (Amstalden et al. 2014). Liver-derived Insulin-like Growth Factor-1 (IGF-1), depends on the nutritional status of the animal and acts as a metabolic indicator at several sites of the hypothalamus-pituitary-ovarian axis (D'Occhio et al. 2019). Therefore, this important metabolic mediator is involved in the onset of puberty in cattle (Cooke et al. 2013; Samadi et al. 2014; Rodríguez-Sánchez et al. 2015).

The attainment of puberty and the onset of ovarian cyclicity can be detected by assays of plasma progesterone (P4) (Terzano et al. 2007) secreted by the corpus luteum. Furthermore, Inhibin-A (Inh-A) concentrations begin to be detectable in blood during the peri-pubertal period (Raivio and Dunkel 2002). The Inhibins are gonadal peptides that selectively and potently inhibit FSH secretion from the pituitary gland, and their concentrations in follicular fluid and blood are correlated with ovarian follicular dynamics. In fact, the Inhibin-A subunit concentrations rise as the follicles approach the preovulatory stage (Medan et al. 2007).

Several trials have been conducted to verify the age at puberty of buffalo heifers, with particular attention to heifer management and nutrition (Barile 2005; Terzano et al. 2007; Campanile et al. 2010). These studies showed that feeding and growth play a central role in achieving the weight required for conception. However, metabolic hormones, linking nutrition and puberty, have not been extensively investigated in buffalo heifers.

Therefore, in order to provide physiological knowledge useful for adequate management practice, the present exploratory study was designed to investigate nutritionally-related hormones (LEP, IGF-1) linked with growth and the onset of puberty in buffalo heifers reared on two different production systems: confinement system feeding with total mixed ration (feedlot), or using pasture as the forage source. As a part of a larger study, the values of age at puberty, body weight and daily weight gain at puberty have been previously published elsewhere (Sabia et al. 2014).

Material and methods

Location, animals, management and diets

The study was carried on at the CREA Research Centre for Animal Production and Aquaculture experimental farm, located near Rome, Italy (42°03′ N, 12°57′ E, 165 m above sea level). Pre-pubertal Mediterranean buffalo heifers (*Bubalus bubalis*) were used in this study, for a period of 14 months (from September 2010 to October 2011, from 8 to 21 months of age). Animals from the same farm and from birth until 8 months of age have been managed under the same farming conditions (group-housed in an indoor slatted floor pen with an outdoor paddock). Then, the buffalo heifers were randomly assigned to two groups, balanced for age (221 ± 11 and 223 ± 11 days, respectively) and body weight (BW) (132 ± 11 and 137 ± 12 Kg, respectively): feedlot reared group (FR, $n = 13$), housed in an indoor slatted floor pen (4.0 m^2/animal) with an outdoor paddock (4.0 m^2/animal) and fed with total mixed ration (TMR); pasture grazing group (PG, $n = 13$), kept on pasture in the same farm, grazing on a fenced Mediterranean natural pasture of 5 ha, which was intended exclusively for this group of experimental animals. Alfalfa hay was offered depending on pasture availability (from December to March). The components and chemical composition of the two diets offered to the animals have been reported in Sabia et al. (2014). Milk Forage Unit (MFU)/Kg dry matter was 0.9 and 0.6 for FR and PG groups, respectively. The pasture had an annual rainfall of about 512 mm and the mean annual environmental temperatures ranged from 5.5 to 26.9 °C. The total available pasture was occupied by annual grass vegetation. Grass availability in dry matter (DM/ha), and chemical and botanical compositions of the pasture were measured, as reported in Sabia et al. (2014). The number of species in the sward was related to the period of grass development. The peak of productivity was recorded in spring and DM availability was greater in spring (1504 Kg/ha) than in November (337 Kg/ha), as expected. The Graminaceae percentage prevailed in November, whereas it decreased in spring when the Leguminosae, Compositae and others were more represented.

The heifers were weighed monthly, in order to measure the BW and the average daily weight gain (DWG). Dry matter intake, crude protein (CP) and MFU ingestion were reported in Sabia et al. (2014). At the end of the trial, body condition score (BCS) was recorded utilising the 1–9 scale of Wagner et al. (1988), modified for the buffalo species by Campanile et al. (1998). Animals were routinely treated with anthelmintics at least twice yearly. Animal health and welfare were carefully monitored during the study period by practitioners designed for the Research Centre.

Blood sampling, hormone assays, puberty assessment

Animals were gathered temporarily before blood sampling, always performed in the early morning. Blood samples were drawn by jugular venipuncture in 10 mL vacuum tubes containing Li-heparin (Venoject, Terumo, Leuven, Belgium), immediately centrifuged at 2000 g for 15 min and plasma was harvested into 2 ml Eppendorf vials, frozen and stored at $-20\,^{\circ}$C until analysed, within 3 months after the completion of samplings.

Starting at 13 months until 21 months of age, plasma LEP and IGF-1 concentrations were determined in blood samples collected monthly. Plasma concentrations of LEP were assayed using a multispecies RIA Kit (Linco Research, Inc., St. Louis MO, USA). The LEP RIA kit was validated for use in buffalo plasma, as previously described by Campanile et al. (2010). The sensitivity of the LEP assay was 0.37 ng/mL and the intra-and inter-assay CV was 5.6 and 8%, respectively. Plasma concentrations of total IGF-1 were determined by an ELISA kit (DRG IGF-1 600600 ELISA, DRG Diagnostics, Marburg, Germany) which is a solid phase enzyme-linked immunosorbent assay, based on the principle of competitive binding. Assay sensitivity was 1.3 ng/mL and intra- and inter-assay CV were 5.7 and 7.5%, respectively.

Starting at 17 months of age, blood samples were collected every 20 days to determine Inh-A, and every 10 days to determine P4 concentrations. Plasma Inh-A concentrations were determined by an ELISA kit (Inhibin A DSL-10-28100, Diagnostic Systems Laboratories Inc, Webster, Texas, USA), previously validated for buffalo species (Todini et al. 2007). Assay sensitivity was 1.0 pg/ml and the intra-and inter-assay CV was 3.9 and 7.1%, respectively. Plasma P4 concentrations were determined by a solid-phase P4 radio-immunoassay kit (Diagnostics Product Corporation, Los Angeles, CA, USA). Intra- and inter-assay CVs were 7.6 and 16.1%, respectively. Assay sensitivity was 0.08 ng/

mL. Heifers were considered to have achieved puberty when plasma P4 concentration exceeded 1 ng/mL for the first time and achieved cyclic ovarian activity when plasma P4 levels exceeded 1 ng/ml for two consecutive samples with a lower intermediate value (Terzano et al. 2007). At the same time, starting at 17 months of age and at 20 days interval, the uterus and ovaries were examined by transrectal ultrasonography (Aloka SSD Prosound 2 scanner, Hitachi Medical System, Buccinasco, Italy, equipped with a 7.5 MHz linear-array transducer), to follow changes in the size of ovaries and in number of follicles and to confirm the achievement of puberty by the presence of the first corpus luteum. After two cycles, all the heifers were naturally mated. Pregnancies were assessed by transrectal ultrasonography each month starting 30 days post-mating and conception rates were calculated as pregnant/mated heifers. The interval from puberty to fertile mating (conception) was also recorded. All mandatory laboratory health and safety procedures were complied with during the conduct of the experimental work reported in this paper.

Statistical analysis

Normal distribution and homogeneity of variance were investigated by Shapiro-Wilk and Levene's test, respectively. Data were then subjected to ANOVA for repeated measures following the GLM procedures of the SAS 9.1.3 Statistical Analysis Software for Windows (2008). The mathematical model for age and weight at puberty, intakes and DWG included one fixed effect due to the rearing system. Mixed model ANOVA was utilised to analyse LEP, IGF-1 and Inh-A. Full factorial models were built including the rearing system (2 levels: FR and PG groups) and time (sampling date, 9 levels) as between- and within-subjects factors, respectively. Greenhouse–Geisser correction was used whether Mauchly's test indicated a violation of sphericity, while Sidak correction was used for pairwise comparisons. Correlations were estimated by means of Pearson's correlation coefficient (CORR procedure). The average body weight of heifers at puberty was predicted from the regression analysis of BW on age. Chi-Squared test was applied to analyse conception rates. Results are presented as means and the variability is expressed as SEM. The significant difference was set at $P < 0.05$.

Results

Progesterone, puberty and conception, INH-A

Age at puberty was not different between the two groups (599.5 ± 8.5 and 610.5 ± 8.9 days, in FR and PG

groups, respectively). Before puberty, P4 mean levels were very low in both FR and PG groups, as expected. At puberty, PG heifers had higher mean plasma P4 than FR heifers (4.90 ± 0.54 vs. 2.40 ± 0.52 ng/mL, respectively; $P = 0.05$). Heifers at pasture had a shorter mean interval from the onset of puberty to conception than the FR group (51.3 ± 5 vs. 67.2 ± 4.8 days, respectively; $P < 0.05$) and the conception rate was not different (83.3 vs. 84.6%, for PG and FR group, respectively).

Plasma Inh-A means concentrations are shown in Figure 1, where the data are temporally referred to the sample relative to puberty onset (time 0). About 1 month before puberty plasma Inh-A began to gradually increase in both groups and this increase continued throughout the peripubertal period. The mean values at puberty were 18.8 ± 3.5 and 13.1 ± 2.9 pg/mL,
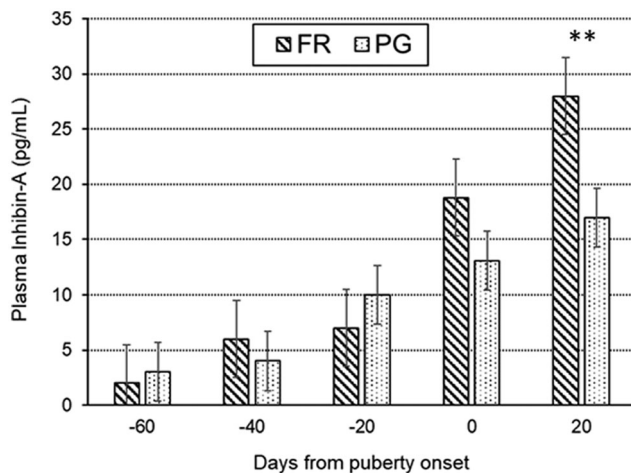

Figure 1. Mean ($\pm$ SEM) plasma Inhibin-A concentrations in buffalo heifers reared in feedlot (FR, $n = 13$) or grazing at pasture (PG, $n = 13$). $** = P < 0.01$ between groups.
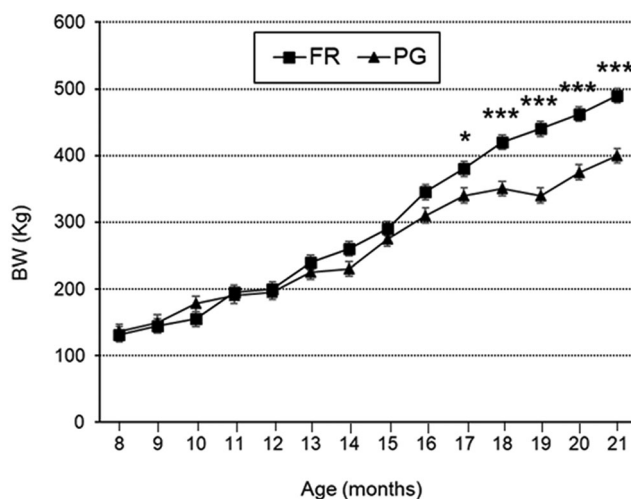
in FR and PG groups, respectively. Twenty days after puberty Inh-A mean concentration was higher in FR than PG heifers. Correlations of Inh-A were positive with IGF-1 ($r = 0.75$; $P = 0.03$) and negative with P4 ($r = -0.41$; $P = 0.04$).

Growth

At puberty, FR heifers were heavier (462 ± 3 Kg, $P < 0.001$) and showed greater mean DWG (0.87 ± 0.01 Kg/day, $P < 0.001$), in comparison with the PG group (375 ± 3 Kg and 0.62 ± 0.01 Kg/day, respectively). From around 17 months of age (10 months after the beginning of the trial, month of June), and forward, the BW of the FR group was higher than that of the PG group (Figure 2). The BW of heifers increased by an average of 24.8 ± 1.33 vs. 18.2 ± 1.62 Kg/month for FR and PG groups, respectively ($P < 0.001$). Both groups reached the peak of DWG (Figure 3). in the March-May period (1.22 ± 0.01 and 1.09 ± 0.01 Kg/day, for FR and PG group, respectively), then decreased during summer (June–August period), when the values were lower in PG than FR group (0.23 ± 0.01 vs. 0.8 ± 0.01 Kg/day, respectively; $P < 0.01$).

The correlation between DWG vs. BW was strong (both groups together: $r = 0.81$ ($P < 0.001$). Age at puberty and DWG were positively correlated in the PG group ($r = -0.55$, $P = 0.05$). At the end of the trial, BCS was higher ($P = 0.01$) in FR (7.65) than in the PG group (6.15).

Leptin and IGF-1

Mean plasma concentrations of LEP and IGF-1 were both affected by rearing systems ($P < 0.05$), being

Figure 2. Mean ($\pm$ SEM) Body Weight (BW) of buffalo heifers reared in feedlot (FR, $n = 13$) or grazing at pasture (PG, $n = 13$). $* = P < 0.05$; $*** = P < 0.001$ between groups.
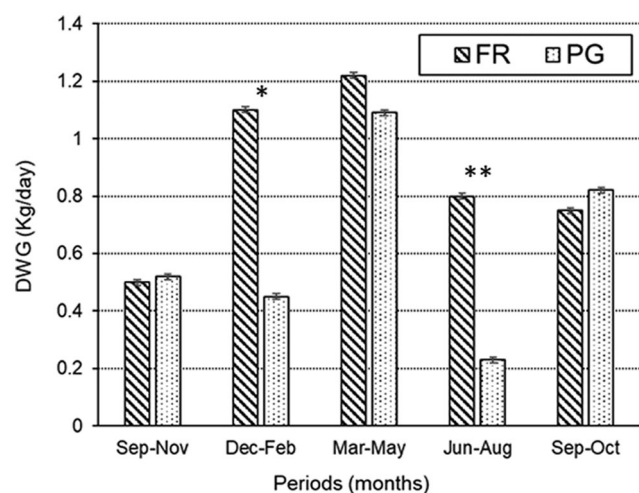

Figure 3. Mean ($\pm$ SEM) Daily Weight Gains (DWG) of buffalo heifers reared in feedlot (FR, $n = 13$) or grazing at pasture (PG, $n = 13$). $* = P < 0.05$; $** = P < 0.01$ between groups.

higher in FR than the PG group. Until 4 months before puberty (16 months old), plasma LEP (Figure 4) was not different between groups (overall mean 4.16 ± 0.82 ng/mL). Afterwards, LEP sharply increased in FR heifers only, becoming higher in these latter than PG heifers, and with rearing system x time interaction ($P < 0.001$). From 18 months of age until the end of the study, plasma LEP means in the FR group were higher than in the previous period ($P < 0.05$). The mean values at puberty were 5.56 ± 0.89 and 2.8 ± 0.5 ng/mL ($P < 0.01$), in FR and PG groups, respectively. Plasma LEP showed positive correlations with BW ($r = 0.52$; $P < 0.001$) and IGF-1 levels ($r = 0.65$; $P < 0.001$).
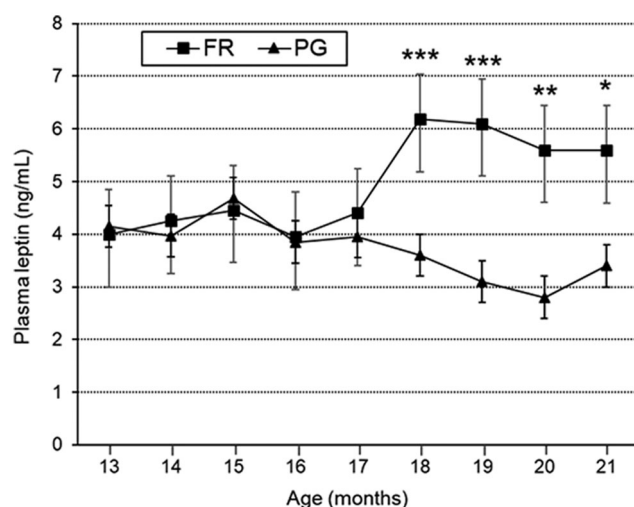

Figure 4. Mean (± SEM) plasma Leptin concentrations in buffalo heifers reared in feedlot (FR, $n = 13$) or grazing at pasture (PG, $n = 13$). * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$ between groups.
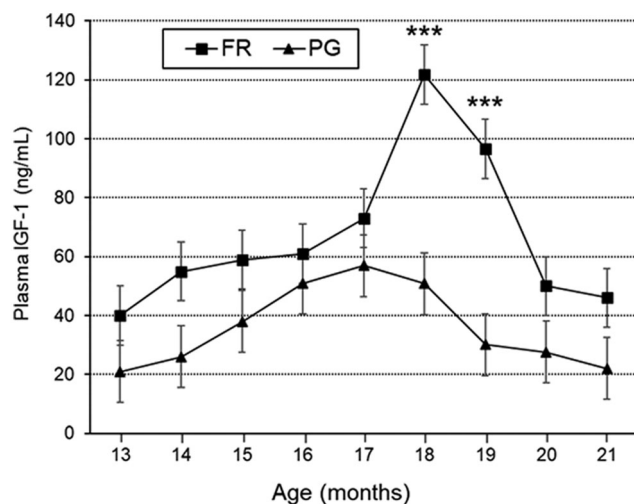

Figure 5. Mean (± SEM) plasma Insulin-like Growth Factor 1 (IGF-1) concentrations in buffalo heifers reared in feedlot (FR, $n = 13$) or grazing at pasture (PG, $n = 13$). *** = $P < 0.001$ between groups.

Plasma IGF-1 (Figure 5) showed patterns similar to those of LEP. Until 3 months before puberty (about 17 months old) the means were not different (57.6 ± 11.1 and 38.6 ± 10.8 ng/mL for FR and PG groups, respectively). Afterwards (2 months before puberty), plasma IGF-1 levels started to increase rapidly in FR heifers only, showing rearing system x time interaction ($P = 0.05$). In the FR group, IGF means were higher at 18 and 19 months of age than at the other sampling dates. Then, mean values at puberty dropped to 50 and 27.6 ng/mL, in FR and PG groups, respectively, with no differences between groups.

Discussion

Growth, puberty and conception

Measurements of Inh-A are a better indicator than Oestradiol of follicular development in buffalo cows (Malfatti et al. 2007) and may also provide a sensitive tool for the assessment of gonadal maturity in buffalo (Terzano et al. 2012). A negative correlation with P4 levels are likely due to the opposite temporal pattern of secretion of the two hormones, in relation to the follicular or luteal phases of the ovarian cycle. A positive correlation with IGF-1 could be ascribed to the stimulating action by this growth factor at the ovary level since systemic IGF-1 is required for follicle development and ovulation (Ginther 2016) and Inh-A concentration increases parallel to follicle size (Malfatti et al. 2007).

As in other species, also in buffaloes, the beginning of cyclic ovarian activity depends on live weight, being a target breeding weight of 55–65% of their adult BW (Terzano et al. 1996). In the current study, FR and PG heifers attained puberty respectively at 73% and 60% of mature body weight, considered to be 630 Kg (Borghese et al. 1996). The rate of BW gains is considered the most important variable affecting age at puberty (Allen et al. 2017). In *Bos indicus* (Samadi et al. 2014; Nepomuceno et al. 2017; Moriel et al. 2020) and beef *Bos taurus* heifers (Heslin et al. 2020), improved nutrition and higher intakes after weaning advanced puberty onset and/or pregnancy rate. Conversely, in the present study, age at puberty and conception rate were not affected by the rearing conditions and by the different DWG and BW. The correlations found between BW, DWG and age at puberty in the two groups, indicate that grazing animals efficiently used the available feed resources (pasture and a limited hay supplementation) for their adequate development. Meanwhile, in the FR group, beyond the satisfaction of growth requirements, spare nutrients were used for

fat mass accumulation, and the surplus of BW was not associated with improved reproductive performances, seeming mostly related to fat deposition, as indicated also by the higher BCS. Furthermore, several reports point up the actual time window in which improved nutrition and increased BW gains are effective in anticipating puberty onset (Perry 2016): advantages have been obtained with enhanced weight gains before weaning (Rodriguez-Sanchez et al. 2015), but not post-weaning (Rodriguez-Sanchez et al. 2018). The critical period during which heifers are more sensitive to the effects of nutrition on pubertal acceleration is plausibly between 4 and 9 (Cardoso et al. 2018) or 4–7 (Amstalden et al. 2014) months of age. The present study started when the heifers of the PG group aged 8 months were put on pasture, therefore when the time-window of nutritional programming of puberty may already passed by. The developmental plasticity at the age of the present study is very low, in comparison with the previous stages of life (van Niekerk et al. 2021). However, comparisons between studies are difficult, firstly due to the very large differences in experimental timing and quality of the diets. In addition, other environmental factors including photoperiod, may play a role (Schillo et al. 1992; Samadi et al. 2014; Ferraz et al. 2018).

Regarding reproductive performances, our findings agree with many previous studies on cattle, in favour of pasture systems (Washburn et al. 2002; Olmos et al. 2009; Mulliniks et al. 2013; Arnott et al. 2017). Exercise is required for adequate reproductive function in cattle, as the lack of motor activity may halter puberty attainment and pregnancy rates, despite adequate growth rates and final BW (Cappellozza et al. 2014).

Leptin and IGF-1

Plasma concentrations of LEP and IGF-1 were both affected by rearing systems. As found in the present study, LEP and IGF resulted positively correlated in Murrah buffalo heifers, but correlations were lost within the groups fed diets with high or low energy levels when separately considered (Di Palo et al. 2005). About four months before puberty (8 months after the start of the study and 16 months of age), LEP sharply increased in FR heifers only, when the BW started to be higher than that of the PG group. Plasma LEP showed positive correlations with BW and IGF-1 levels, as buffalo cows with greater BCS had higher plasma LEP levels (Campanile et al. 2010). Higher blood LEP usually accompanies improved nutrition, higher BCS, BW and BW gains (Samadi et al. 2014; Allen et al. 2017; Bruinjé

et al. 2021), closely associated with pubertal progression (Garcia et al. 2002). On the other hand, previous studies report no association of plasma LEP and diets in growing heifers (Ferraz et al. 2018; Rodríguez-Sánchez et al. 2018). Plasma LEP levels strongly correlated with cumulative BW gains, more than fluctuations of BW, in Murrah buffalo heifers (Di Palo et al. 2005). Interestingly, in that study, overall LEP correlated with BCS, but not for higher values of BCS (from good to high). Authors suggested that, at higher feeding levels, fat storage is in proportion more abundant in the abdominal depots than in the subcutaneous adipose tissue. Since leptin is mainly secreted by the subcutaneous adipose tissue, the LEP plasma levels do not increase proportionally to high rates of overall fat accretion. Therefore, the amount of fat storage plays the main role in the levels of plasma LEP, but also the kind and the localizations of these depots are determinants to modulate LEP circulating levels (Di Palo et al. 2005). The function of LEP as an indicator of growth and reproductive development of heifers seems less clear and secondary than that of IGF-1 (Rodríguez-Sánchez et al. 2015). Leptin itself does not trigger puberty but is a permissive yet critical signal for puberty onset (Garcia et al. 2002; Roa et al. 2010). A certain threshold of LEP levels is necessary for puberty attainment (Cooke et al. 2013), communicating at the central level that the energy availability is sufficient to support the transition to puberty. In the present trial, only FR heifers showed a rise in LEP levels, but PG heifers attained puberty at the same time, letting to conclude that in these latter the feeding regimen was sufficient to ensure adequate development and adiposity, and the threshold of LEP was reached, anyway. Similar to the LEP pattern, approaching puberty, plasma IGF-1 levels started to increase rapidly in FR heifers only. The finding of greater plasma IGF-1 concentrations in heifers of the FR group is consistent with previous studies about relationships between nutrition, BCS and IGF-1 in buffalo cows (Campanile et al. 2010). Elevated IGF-1 levels were related to improved nutrition also in beef (Rodriguez-Sanchez et al. 2015; Rodriguez-Sanchez et al. 2018), as well as in Brahman (Samadi et al. 2014) and crossbreed (Moriel et al. 2020) heifers. Previous research (Guggeri et al. 2014; Samadi et al. 2014; Rodriguez-Sanchez et al. 2015) report negative correlations between IGF-1 levels and age at puberty. However, the nutritional-linked rise of IGF-1 levels, which was correlated with advanced puberty, was observed at weaning (6 months of age: Rodriguez-Sanchez et al. 2015), or about one year before puberty (12–13 months of age: Samadi et al. 2014), thus well before the increase observed in FR

heifers of the present trial (18 months of age). Again, we can hypothesise that the potential effects of nutrition and associated systemic IGF-1 levels on timing the onset of puberty, may take place at the early stages of development, before the age at which the present trial started (van Niekerk et al. 2021). Furthermore, it was proposed that *Bos taurus* and *Bos indicus* cattle may differ in the effects of insulin and IGF-1 on reproductive function (Sartori et al. 2016; D'Occhio et al. 2019). Breed (Rodriguez-Sanchez et al. 2018) and intra-breed genetic effects (Ferraz et al. 2018) have also been reported in this regard.

Conclusions

The results of this trial suggest that buffalo heifers at pasture (PG) experienced adequate growth rates to reach puberty at the same age as the feedlot ones (FR), despite blood LEP and IGF-1 being higher in FR heifers. Increased plasma hormone levels can be attributed to the higher amount of energy ingested by FR, but not to a factual energy deficit in PG ones, at least not to a degree that compromise adequate sexual development. For PG heifers, DWG was important to attain puberty, whereas in the FR group, after requirements for development were met, further nutrients were only used for fat mass accumulation. Furthermore, heifers at pasture had shorter puberty to conception intervals than those reared in the feedlot, and the pregnancy rates at the first mating season were similar. Positive relationships were found between peripheral concentrations of leptin and body weight and between leptin and IGF-1, confirming that these hormones can be considered objective indicators of the energetic status, also in growing buffalo heifers.

Acknowledgements

Ethical approval

The study was planned and performed at the CREA Research Centre for Animal Production and Aquaculture experimental farm, authorised to use farm animals for experimental design (as stated in DM 26/96-4 of Italian Health and Welfare Ministry). Although predating the transposition of European Directive 2010/63/EU into Italian law (Legislative Decree 26/ 2014), the experimental design was carried out according to good veterinary practices under farm conditions. The care and the handling of the animals were in accordance with the Italian law on the protection of animals used for experimental and other scientific purposes, in force at that time (DL n.116/1992) and followed the European recommendations for the protection of experimental animals (EU Directive 2010/63/EU). The animals were supervised by the responsible of animal welfare and the designated veterinarian as required by the law in force at the time of the trial.

Disclosure statement

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Data availability statement

The data associated with this paper are available from the Authors, upon reasonable request.

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