

## TITLE

**Application of longitudinal data analysis allows to detect differences in pre-breeding growing curves of 24-month calving Angus heifers under two pasture-based system with differential puberty onset.**

## AUTHORS

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## ABSTRACT

**Background.** Longitudinal data analysis contributes to detect differences in the growing curve by exploiting all the information involved in repeated measurements, allowing to distinguish changes over time within individuals, from differences in the baseline levels among groups. In this research longitudinal and cross-sectional analysis were compared to evaluate differences in growth in Angus heifers under two different grazing conditions, *ad libitum* (AG) and controlled (CG) to gain 0.5 kg/day.

**Results.** Longitudinal mixed models show differences in growing curves parameters between grazing conditions, that were not detected by cross sectional analysis. Differences ( $P < 0.05$ ) in first derivative of growth curves (daily gain) until 289 days were observed between treatments, being AG higher than CG. Correspondingly, pubertal heifer proportion was also higher in AG at the end of rearing (AG 0.94; CG 0.67).

**Conclusion.** In longitudinal studies, the power to detect differences between groups increases by exploiting the whole information of repeated measures, modelling the relation between measurements performed on the same individual. Under a proper analysis valid conclusion can be drawn with less animals in the trial, improving animal welfare and reducing investigation costs.

**Key words:** puberty, heifer, grazing, rearing, growth, longitudinal data.

## INTRODUCTION

While longitudinal and cross-sectional analysis can be used to address the same questions, the first one uses all the information contained in the repeated measurements through time. The defining feature of a longitudinal data set are the repeated observations on individuals, which enables to study changes in the trait within individual, considering the existing correlation in a set of observations on one subject. Hence, the possibility to distinguishing changes over time within individuals, from differences among groups in their baseline levels<sup>1</sup>, allows to exclude some variation within treatment from the error term<sup>2</sup>, and this is exploited to draw more powerful inferences. This kind of longitudinal approaches have been used in animals to detect day to day growth and body composition differences<sup>3-5</sup>.

The use of repeated measurements allows to conclude about the process, rather than the evaluation of the cumulative result (i.e. cross-sectional). When correlations in longitudinal data set are considered and all measurements are taken into account to estimate the regression coefficients (like the Average Daily Gain, ADG) the results are more accurate and get protection against biases caused by missing or wrongly collected data<sup>1</sup>. Additionally, modelling of covariance between random effects (for example the relationships between related animals in the trial), aid to correct the estimation of fixed effects<sup>6</sup>. Ignoring these sources of variation forces to employ a higher number of animals in the study to detect an effect or test an hypothesis<sup>2</sup>.

Females reproductive efficiency is a major contributor to the profitability in cow-calf systems. Namely, first calving at 24 months of age allows an extra calf in the productive life of the female. This helps to decrease the proportion of unproductive animals in the herd, and therefore release areas that can be occupied by productive cows<sup>7,8</sup>. Additionally, early calving within the calving season, allows to wean a heavier (older) calf and extend the period until the next mating season<sup>9,10</sup>. For a successful breeding season, all females should arrive to the first

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estrous cycling period before mating. Indeed has been proposed that puberty should be reached 45 days before the breeding season<sup>11-13</sup>. This developmental reproductive milestone would be influenced by the productive system, female physiology and genetics<sup>9,14,15</sup>, but the main factor that can be handled is nutrition under rearing.

Growing of rearing heifers is often evaluated through comparisons of final live weight or average daily gain (ADG), which leads to ignore the study of individual change and underutilize the collected information. However, as an average, ADG does not account for differences in daily growth or restrictions in short periods during rearing, that could have an impact on reproductive development. Observations in pasture-based rearing have variation in composition and digestibility along the year according to environmental conditions, which could lead to transitory nutritional restrictions<sup>16,17</sup>. Hence, longitudinal analysis could provide more efficient estimators to test differences in the growth curve rather than cross-sectional analysis<sup>2</sup>, that could be related to those nutritional variations.

The aim of this study was to evaluate the capability of longitudinal and cross-sectional analysis to detect differences in growth of Angus heifers under two pasture based rearing nutritional conditions. Furthermore, a possible relationship between growth difference and puberty at beginning of breeding season was analyzed, under the hypothesis that longitudinal models can detect local differences between growing curves, and those differences could be related with pubertal status at breeding season.

## MATERIAL AND METHODS

### *Animal resources, feeding conditions and phenotypic data*

The study included 468 repeated measurements obtained from 36 weaned Angus heifers born between July 27<sup>th</sup> and September 29<sup>th</sup>, 2012 at the National University of La Plata Experimental Farm “El Amanecer” (Magdalena, Buenos Aires, Argentina 35°15’S – 57°37’O). Every procedure conducted on an animal was reviewed and approved by the National University of La Plata School of Veterinary Sciences Institutional Review Board on Animal Use and Care [CICUAL, for its Spanish acronym (Protocol n° 74-4-18T)]. Heifers were weaned on March 1st, 2013. At the beginning of the study, 55 days after weaning, heifers ( $248 \pm 17$  days,  $167 \pm 21$  kg) were randomly assigned to two rearing treatments: *ad libitum* grazing (AG; n=18) and controlled grazing (CG; n=18), for 182-day from April 4th until October 10th, 2013. Both treatments were based on winter species pasture, which was obtained by modifying wet grasslands into a winter grass resource using herbicides to promote germination and establishment of winter annual species, mainly *Lolium multiflorum*, *Bromus catharticus* and *Gaudinia fragilis*.

Forage availability in both treatments was monitored every  $14 \pm 1$  days by means of the grass offer in dry matter (DM, kg per hectare). Briefly, at each measurement day, four 0.5 m-side square areas were randomly selected for each treatment and all the grass delimited was clipped and weighed within one hour. Then, each grass sample was dried separately in an air circulation oven at 60 °C until constant weight was reached. Finally, those weights were used to determine DM percentage in the forage to be eaten and in kg per hectare. The forage availability in AG treatment was calculated to guarantee that heifers would not experience any intake restriction during the trial. The expected intake for the AG group in the whole trial was 21,150 kg of DM, then an area of 6 hectares was assigned which would offer between 24,000 and 30,000 kg of DM within the period of the trial. Even though, the remaining offer was monitored as set before. The

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grazing area for CG was assigned and renewed every  $14 \pm 1$  days to guarantee a 60% of adult weight at the end of the rearing (258 kg), considered as a target weight. The new paddock area was determined taking into account the measured DM forage offer (Supplementary Table 1) (assuming that half of the available forage will be grazed by the heifers), and an expected forage intake according to NRC for heifers of the previously measured live weight and an ADG = 0.5 kg/day.

Phenotypic data were recorded every  $14 \pm 1$  days during the rearing test period. Measurements ( $n = 13$  for each animal) included live weight and ultrasonographical ovarian activity using a 7.5 MHz linear-array transducer connected to a B-mode scanner (Aquila vet, Esaote SpA, Genova, Italy). An animal was considered to have reached puberty when a corpus luteum could be identified for the first time<sup>18,19</sup>.

#### *Estimated energy balance (EB) during the trial*

After the trial, EB of each animal for the following inter-measurement period ( $14 \pm 1$  days) was estimated considering the offered energy and the predicted requirements of the animals for the period (EB = offered energy – energy requirements). Offered energy was defined for each animal at the beginning of an inter-measurement period, and was estimated using the equivalence reported by Guaita and Fernandez<sup>20</sup>. Those authors reported the MJ of metabolizable energy per kg of DM of forage offered for similar pastures in the same biosphere where the trial was performed, the values 11.7 MJ/kg DM and 11.3 MJ/kg DM were used for autumn and winter periods, respectively. Thus, DM availability in the whole grazing area on the day of measurements and of the new parcel were considered for AG and CG, respectively. The required metabolizable energy (Req) was estimated by testing weight and ADG linear and quadratic multiple regressions to fit NRC table data<sup>21</sup> (Supplementary Table 1) to obtain a predictive equation for the energy requirements of an animal depending on live weight and ADG in the previous period. REG

procedure of SAS<sup>®</sup>9.4 (2012) was used to evaluate four different models to fit weight data between 200 and 400 kg and ADG data between 0-1 kg/day. Finally, the most parsimonious equation ( $r^2=0.96$ ) was used to calculate the requirements

$$Req = -2.532 + 0.043 * W + 12.786 * ADG - 3.953 * ADG^2.$$

#### *Statistical analysis*

In both groups, ANOVA procedure implemented in SAS<sup>®</sup> 9.4 was performed to check the randomized animal assignment to each treatment according to initial weight and age, and to look for differences in age and weight at puberty and final weight between treatments. Differences between groups were calculated using Bonferroni correction.

Weight longitudinal data (234 registries for each treatment) were analyzed by fitting a mixed-effect regression model<sup>2</sup> which allows to use the whole information included in correlated registries and to correct the effect of genetic relatedness between animals. Let  $y_{ijkt}$  represent the  $k_{th}$  weight of individual  $i$  in the  $j$  grazing treatment at  $t$  age,

$$y_{ijkt} = g(t)_j + r(\delta, t)_i + e_{ijkt} \quad [1]$$

where  $g(t)_j$  accounts for the mean weight trajectory of all animals in the  $j$ th grazing treatment, defined by the following quadratic polynomial,

$$g(t)_j = \alpha_{0j} + t\alpha_{1j} + t^2\alpha_{2j}$$

and  $r(\delta, t)_i$  accounts for a quadratic random regression function associated to the  $i$ th animal, defined as

$$r(\delta, t)_i = t\delta_{1i} + t^2\delta_{2i}$$

where,  $\delta_{1i}$  and  $\delta_{2i}$  denote the linear and quadratic random coefficients on the age ( $t$ ) for the  $i$ th animal.

Note that this function models the individual deviations around the mean phenotypic trajectory. The following covariance structure was assumed for the random regression function,

$$\begin{bmatrix} \text{Var}(\delta_{1i}) & \text{Cov}(\delta_{1i}, \delta_{2i}) \\ \text{Cov}(\delta_{2i}, \delta_{1i}) & \text{Var}(\delta_{2i}) \end{bmatrix} \otimes \mathbf{A}$$

where  $\mathbf{A}$  is the additive relationships matrix and ' $\otimes$ ' stands for the Kronecker operator.

Finally,  $e_{ijkt}$  stands for an independent normally distributed error.

A second analysis was performed to test differences in live weight curves of pubertal vs non-pubertal animals, using a similar model:

$$y_{ijkt} = x_i\beta + g(t)_j + r(\delta, t) + e_{ijkt} \quad [2]$$

where  $y_{ijkt}$  represents the  $k_{th}$  live weight of individual  $i$  in the  $j$  pubertal classificatory level (pubertal or non-pubertal) at the  $t$  age, and a time-independent fixed effect was included to consider the treatment effect; hence  $\beta$  is a parameter vector for fixed effects of treatment (2 levels), and  $x_i$  is the corresponding incidence vector for the  $i_{th}$  individual. Then, the  $g(t)_j$  function is an analogous quadratic polynomial, in this case accounting for the average live weight curve of either pubertal or non-pubertal animals. Lastly,  $r(\delta, t)_i$  stands for the quadratic random regression function associated to the  $i_{th}$  animal, defined as before, and  $e_{ijkt}$  for an independent normally distributed error.

The first derivative of regressions was obtained for equation 1 and 2, to study the change considering age and additive relationships correction and randomizing the sampling error (mainly gastrointestinal fill).

Equations 1 and 2 were fitted using SAS® 9.4 software. The INBREED procedure was used to compute the covariance between relative animals (A Matrix), and the Restricted Maximum Likelihood (REML), implemented in the MIXED procedure, to estimate the covariance components. Differences between weight along age for both grazing treatments and



pubertal status were estimated setting linear contrasts at day by day using ESTIMATE statement, as well as derivative daily gain (dDG) as the first derivative of equations 1 and 2. Interaction between pubertal status and rearing treatment was tested and resulted non-significant ( $P > 0.05$ ), then was not considered in the model to avoid over-parametrization.

## RESULTS

The mean results obtained for both grazing treatment groups and for all animals are presented in Table 1. When all the heifers that reached puberty are considered (29 out of 36), mean age at puberty was  $391 \pm 30$  days and their weight was  $256 \pm 23.8$  kg (Table 1), while on the last sampling (end of rearing and beginning of breeding period), heifers that reached puberty were on mean 22 kg heavier ( $P < 0.05$ ) and 5 days younger ( $P > 0.1$ ), compared with non-pubertal ones (Table 2). Nevertheless, live weight and age at breeding showed no differences between animals in the two grazing rearing treatments in a cross-sectional approach (ANOVA). However, the number of pubertal heifers were different ( $P < 0.05$ ) at the end of rearing: 17 for AG and 12 for CG from 18 in each group (Table 2).

In contrast, longitudinal weight analysis shows significant differences ( $P < 0.05$ ) in the parameters ( $\alpha_i$ ) of the growth curves between grazing treatments (Table 3, Figure 1). However, the estimates for the mean live weight at each age were not significant between treatments. Nevertheless, the estimated daily gain (dDG), as the first derivative of the growth curve, was significantly different between treatments ( $P < 0.05$ ) for 39 days in trial, from 250 days until 289 days of age (Figure 1), being greater for AG.

Additionally, pubertal vs. non-pubertal animals were studied for both treatments. Differences between pubertal and no pubertal heifers were observed using a cross sectional approach, comparing ADG from birth to weaning and during rearing, as well as final live weight (Table 2). Weight curves also showed significant differences ( $P < 0.05$ ) in linear and quadratic parameters (Table 3, Figure 2). Moreover, daily estimates of live weight were different after 287 days of age between groups, at that date heifers that end up reaching puberty during the trial were 6.1 kg heavier than the ones that were still non-pubertal at the end of the study (Figure 2).

Estimated dDG was also significantly different between pubertal and non-pubertal heifers ( $P < 0.05$ ) from 257 days of age, and greater in pubertal heifers (Figure 2).

To have additional information on the nutritional level of each group during the assay, EB was estimated every two weeks. AG showed a persistent EB reduction during the study, but there was no evidence that it was negative in any period. Although EB in the CG remained almost stable, in a short period around 115 and 135 days of rearing (350 and 370 days of age), EB turned negative. Coincidentally, that period overlapped with an increase in the number of pubertal heifers in AG, while in CG no new pubertal heifers were observed until 380 days of age when the energy balance turned positive again (Figure 3).

To have a more classical perspective of the rearing performance of the whole animals and at individual level, a typical growth evaluation of preweaning ADG and rearing ADG for each animal were calculated (Figure 4). All heifers had an ADG greater than 0.40 kg/day in both periods, nevertheless, in both treatments, animals with preweaning ADG greater than 0.75 kg/day or rearing ADG greater than 0.65 kg/day, reached puberty before the breeding season.

## DISCUSSION

The study was performed to test whether longitudinal analysis provides more information than cross sectional analysis, specifically if a longitudinal mixed model can detect differences that were not evidenced with ANOVA. Longitudinal analysis demonstrates growth differences between treatments, namely, the estimated weight curve parameters were different between them (Table 3). Furthermore, dDG was significantly different up to 289 days of age, corresponding to the first 54 days on trial. In this sense, even ADG in the whole rearing was similar for both treatments and the mean weight at any age was not significantly different, each treatment curve was different (Figure 1; Table 3). These differences highlight the gain in information when the analysis includes a focus on the process rather than the endpoint. Analysis to the endpoint (final result) is normally the one considered when setting targets for longitudinal traits in domestic animals<sup>22</sup>. An important aspect to *refine* statistical inference of animal dataset is the capability of reach the same conclusions with a *reduced* number of animal in the trial/model, according with two of the three Rs principles<sup>23</sup>, which leads to both a decrease in expenses and ensuring compliance with ethical principles for animal research, mainly in preliminary trials.

The effect of nutrition over growth have been largely studied by the differences in final live weight, which induces differences in pubertal heifers at breeding<sup>24,25</sup>. In our study, growth differences were detected for the nutritional treatments in the shape of growing curves (Table 3). Differences between pubertal and no pubertal heifers (considering both treatments) at breeding were detected by both approaches, longitudinal and cross sectional, showing that when the differences are large, both methods are powerful enough (Figure 2; Table 2). Even though, the comparison of treatment groups demonstrates that all information that could contribute to the model would reduce the error term and promote an increase in the power of the test. Hence, in this study, longitudinal model detected growth differences between treatments, not detected by

the cross-sectional one, could be related to the differences in the number of pubertal heifers observed at the beginning of the breeding season between them (Figure 1, C).

Having found differences in the number of pubertal heifers and in growth curves between treatments, we searched about possible factors that could be related with these differences. Dry matter availability was measured for each treatment every 14 days, and the EB was obtained for each treatment within each two-week period (Figure 3). It is possible that neutral or negative EB of CG animals would be related with differences in the accumulated proportion of pubertal heifers between treatments. Furthermore, when overlapping the rearing periods were differences in dDG were detected between treatments, with the EB and accumulated puberty (Figure 3), there was a negative EB in the CG group that happened when the firsts heifers starts her sexual activity. These could lead to a speculation that feeding conditions have caused the difference observed in growth and puberty, while to completely test that hypothesis a more complex nutritional response analysis would be needed.

The relationship between rearing nutrition and puberty attainment has been discussed in several studies concluding that under nutrition results in lighter weights and less pubertal heifers at breeding than well-nourished heifers<sup>24,26,27</sup>. In our study there was no differences in final live weight, but the growth pattern through rearing was different between treatments as well the number of pubertal heifers (Figure 1), we speculate by overlapping growth curves with EB about the possible relationship between nutrition, weight and puberty. Furthermore, pubertal and non-pubertal heifers at breeding had differences in growth curve and breeding weight (Table 2; Figure 3). Even though the impact of feeding over growth and puberty has already been demonstrated<sup>19,28-30</sup>, the idea that in grazing conditions, a short fasting period could delay puberty attainment, needs to be tested.

Finally, the effect of growing performance within different growing periods and puberty attainment was visually observed (Figure 4). All heifers with preweaning ADG  $> 0.45$  kg/day and rearing ADG  $> 0.54$  kg/day reached puberty before breeding season, also heifers who exceeded  $0.75$  kg/day in the preweaning period attained puberty during rearing independently of ADG postweaning, in our conditions ( $> 0.5$  kg/day), the effect on early nutrition on puberty. On the other hand, a greater post weaning ADG can reduce the negative conditioning effects of low preweaning ADG on age at puberty. Other observations were that mean age at puberty was 391 days and mean weight was 256 kg, which represents 60% of the mature cow weight in this herd (430 kg) and was similar to the results reported by Morris and Wilson<sup>31</sup>. Actually, 50% of adult weight at breeding have been proposed as target<sup>33</sup>, but in our conditions only one heifer per treatment reach puberty at 215 kg. Probably in grazing systems, which is based on low-cost feedstuff, the traditional target of 65% of cow adult weight remains to be a safety goal to be achieved before matting.

## CONCLUSION

Differences in growth curves were identified in heifers reared on two different grazing systems using longitudinal analysis, these differences could not have been proven by cross sectional analysis. Furthermore, the results suggest that the growing curve affects the amount of cycling heifers at the breeding season, even reaching the same live weight at that time.

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## FIGURE CAPTIONS

**Figure 1:** The figure presents the growth curves and the number of pubertal heifers for both rearing treatments during the trial. (A) live weight (LW); (B) derivative daily gain (dDG) as the first derivative of weight curve; (C) proportion of pubertal heifers (APH).

### FIGURE 1

**References:** live weight in *ad libitum* grazing treatment (LW-AG); live weight in controlled grazing treatment (LW-CG); derivative daily gain in *ad libitum* grazing treatment (dDG-AG); derivative daily gain in controlled grazing treatment (dDG-CG), accumulated proportion of pubertal heifers in *ad libitum* grazing treatment (APH-AG); accumulated proportion of pubertal heifers in controlled grazing treatment (APH-CG).

**Figure 2:** Comparison of the estimated growing curves between pubertal (P) and non-pubertal (NP) heifers during rearing. (A) live weight (LW); (B) derivative daily gain (dDG) as the first derivative of weight curve.

**FIGURE 2**

**References:** live weight in pubertal heifers (LW-P); live weight in non-pubertal heifers (LW-NP); derivative daily gain in pubertal heifers (dDG-P); derivative daily gain in non-pubertal heifers (dDG-NP).

**Figure 3.** (A) Energy balance (EB) and (B) accumulated proportion of pubertal heifers (APH) observed during trial.

### FIGURE 3

**References:** energy balance in *ad libitum* grazing treatment (EB-AG); energy balance in controlled grazing treatment (EB-CG); derivative daily gain in *ad libitum* grazing treatment (dDG-AD); derivative daily gain in controlled grazing treatment (dDG-AC); accumulated proportion of pubertal heifers in *ad libitum* grazing treatment (APH-AG); accumulated proportion of pubertal heifers in controlled grazing treatment (APH-CG).

**Figure 4.** Prewaning and rearing average daily gain (ADG) in pubertal and non-pubertal heifers for each treatment.

**FIGURE 4**

**References:** Pubertal heifers in *ad libitum* grazing treatment (P-AG); non-pubertal heifers in *ad libitum* grazing treatment (NP-AG); Pubertal heifers in controlled grazing treatment (P-CG); non-pubertal heifers in controlled grazing treatment (NP-CG).



**Table 1.** Mean weight, age and average daily gain (ADG) ( $\pm$  SD) of heifers under both grazing rearing treatments at the beginning, at puberty onset and at the end of the study (none of them were significant).

Treatment	N	Weaning age (d)	Weaning weight (kg)	Initial age (d)	Initial weight (kg)	Age at puberty (d)*	Weight at puberty (kg)*	Final Weight (kg)	ADG (kg/day)
AG	18	192 $\pm$ 15	167 $\pm$ 23.7	247 $\pm$ 9	169 $\pm$ 21.6	388 $\pm$ 24	255 $\pm$ 21.8	268 $\pm$ 25.9	0.589 $\pm$ 0.116
CG	18	194 $\pm$ 19	162 $\pm$ 25.2	249 $\pm$ 12	165 $\pm$ 20.1	395 $\pm$ 40	258 $\pm$ 29.4	264 $\pm$ 16.5	0.588 $\pm$ 0.089
Total	36	193 $\pm$ 17	164 $\pm$ 24.2	248 $\pm$ 17	167 $\pm$ 20.7	391 $\pm$ 30	256 $\pm$ 23.8	266 $\pm$ 23.7	0.589 $\pm$ 0.102

\* These values were obtained using data from heifers that reach puberty during the trial: N (AG) = 17, N (CG) = 12

**Table 2.** Mean weight, age and average daily gain (ADG) ( $\pm$  SD) of pubertal and non-pubertal heifers under both grazing rearing treatments.

Treatment	Puberty status	N	Final age (d)*	Final Weight (kg)*	Prewaning ADG (kg/day)	Weaning – rearing ADG (kg/day)	rearing ADG (kg/day)
AG	Pubertal	17	414 $\pm$ 15	268 $\pm$ 21.0	0.725 $\pm$ 0.090	-0.035 $\pm$ 0.327	0.598 $\pm$ 0.113
	Non-pubertal	1	427 $\pm$ 0	259 $\pm$ 0.0	0.714 $\pm$ 0.000	0.054 $\pm$ 0.000	0.440 $\pm$ 0.000
CG	Pubertal	12	416 $\pm$ 20	272 $\pm$ 23.9	0.736 $\pm$ 0.111	-0.030 $\pm$ 0.311	0.623 $\pm$ 0.073A
	Non-pubertal	6	419 $\pm$ 21	247 $\pm$ 26.3	0.620 $\pm$ 0.117	0.149 $\pm$ 0.263	0.520 $\pm$ 0.083B
TOTAL	Pubertal	29	415 $\pm$ 17	270 $\pm$ 21.9a	0.725 $\pm$ 0.102a	-0.014 $\pm$ 0.344	0.608 $\pm$ 0.097A
	Non-pubertal	7	420 $\pm$ 20	248 $\pm$ 24.5b	0.635 $\pm$ 0.114b	0.136 $\pm$ 0.243	0.509 $\pm$ 0.081B

\* at the beginning of the breeding season (end of the experiment)

**Note:** Different letters indicate significant differences (within treatment). Uppercase letters,  $P < 0.01$ ; lowercase letters,  $P < 0.05$

**Table 3.** Estimates of parameters in growth curve analyzed between treatments and between pubertal status.

Effect	Treatment		Pubertal Status		
	AG	CG	P	NP	
$\alpha_0$	-74.68 <sup>a</sup>	-3.56 <sup>b</sup>	AG	-40.41	-4.98
			CG	-42.66	-7.24
$\alpha_1$	1.155 <sup>A</sup>	0.696 <sup>B</sup>		0.946 <sup>A</sup>	0.711 <sup>B</sup>
$\alpha_2$	-0.00081 <sup>A</sup>	-0.00014 <sup>B</sup>		-0.00048 <sup>a</sup>	-0.00025 <sup>b</sup>

**Note:** Different letters indicates significant differences (within analysis and effect). Uppercase letters,  $P < 0.01$ ; lowercase letters,  $P < 0.05$ .

**References:** In treatment: *ad-libitum* grazing (AG); Control grazing (CG); in Pubertal Status: Pubertal (P); (NP) non-pubertal (NP) heifers at breeding season.

FIGURE 1

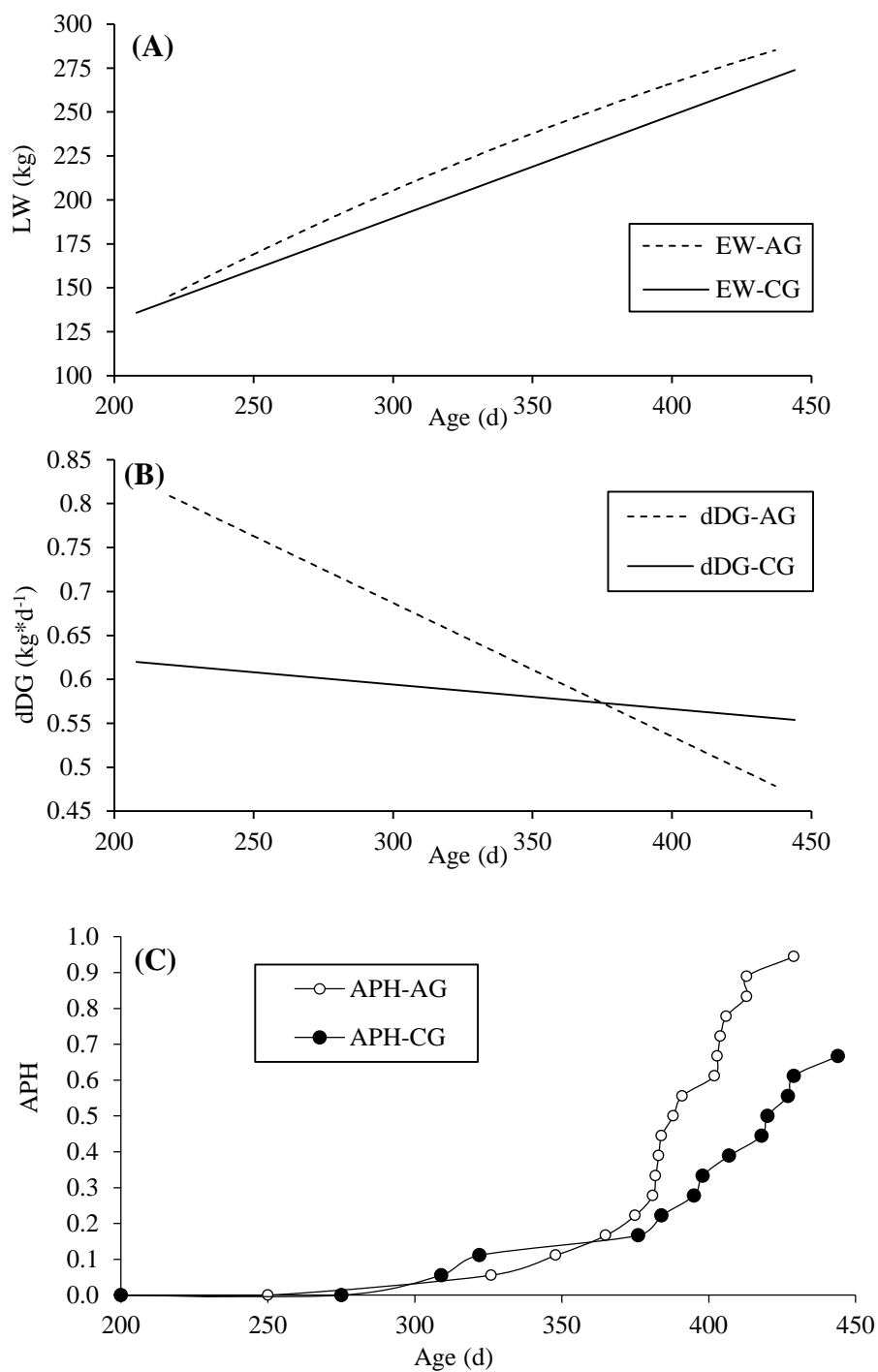


FIGURE 2

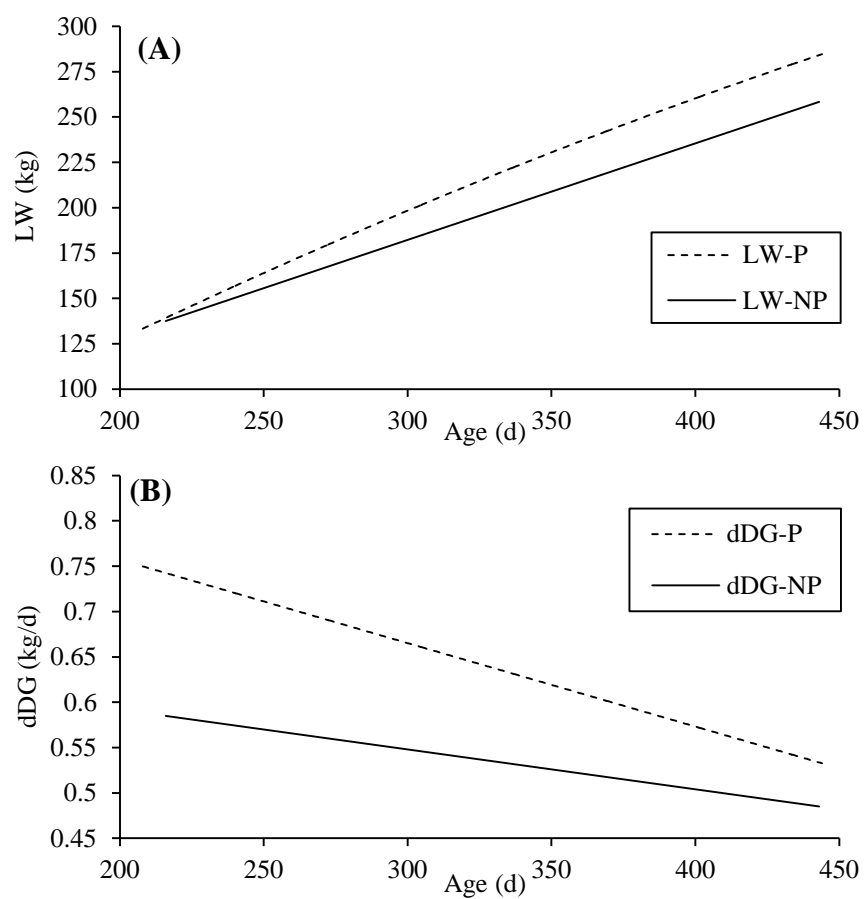


FIGURE 3

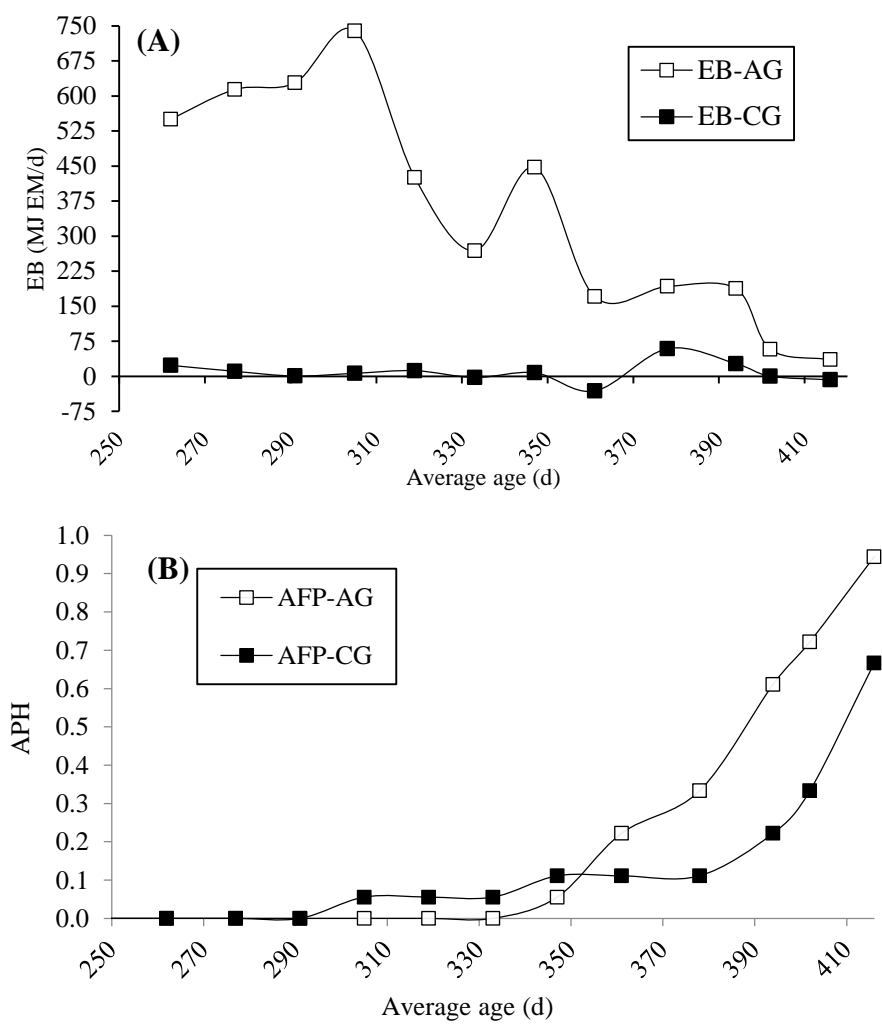


FIGURE 4

