Multivariate analysis about causes of growth delay in early weaned calves *

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Abstract

Coppo, J.A.: ***Multivariate analysis about causes of growth delay in early weaned calves.*** *Rev. vet. 18: 1, 37–45, 2007.* The purpose of this trial was to check if early weaned calf growth delay is due to stress or inadequate feeding. In 4 successive years, assays of 120 days each were carried out, using in total 120 half-bred Zebu calves (2 months old) grazed on natural pasture. Sixty animals were controls fed on maternal milk (C), and other 60 made up the experimental group (E), which was submitted to early weaning and supplemented with balanced food. Weightings and blood extractions were made in days 0, 7, 14, 21, 28, 60, 90 and 120. Hematological and biochemical determinations (42 parameters) included stress and malnutrition indicators, which were evaluated by radio-immune-assay, chemoluminiscency, spectrophotometry, electrophoresis, and electronic particle recount. Such procedures generated more than 40,000 data, which were statistically processed by multivariate techniques, to eliminate the additive effect of random intervention probability (alpha error), characteristic from univariate methods that comprise numerous dependent variables. Variables that obtained significant differences for treatment and/or time effects in repeated measures univariate techniques, were selected for analysis of principal components. Four orthogonal supervariables, susceptible to stress, malnutrition and ontogeny, were created. Weight gains were 79.9 kg (666 g/animal/day) in C, and 61.6 kg (513 g/animal/day) in E. Variations of cortisol, aldosterone, fructosamine, glucose, sodium, potassium, leukocytes, gamma globulins and enzymes suggested absence of stress, examined under an ontogenic frame. On the other hand, decrease of protein, carbohydrate, lipid, and mineral nutritional indicators, revealed malnutrition in E. Selected principal components indicated that most of total variance was due to malnutrition and ontogeny, but not to stress. It is important to develop more appropriate balanced supplements for early weaned calves.

Key words: half-bred Zebu calf, liveweight gain, biochemical alterations, stress, malnutrition.

Resumen

Coppo, J.A.: ***Análisis multivariado de las causas del retraso de crecimiento en terneros de destete precoz.*** *Rev. vet. 18: 1, 37–45, 2007.* Para comprobar si el retraso del crecimiento del ternero precozmente destetado se debe a estrés o inadecuada alimentación, en 4 años sucesivos fueron realizados ensayos de 120 días de duración, empleando en total 120 terneros cruza cebú de dos meses de edad mantenidos sobre pastura natural. Sesenta animales actuaron como controles (C) amamantados al pie de madre y otros 60 constituyeron el lote experimental (E), siendo sometidos a destete precoz y suplementados con alimento balanceado. Durante los días 0, 7, 14, 21, 28, 60, 90 y 120, los terneros fueron objeto de pesajes y determinaciones hematológicas y bioquímicas (42 parámetros) que incluyeron indicadores de estrés e hiponutrición, evaluados por radioinmunoanálisis, quimioluminiscencia, espectrofotometría, electroforesis y recuento electrónico de partículas. Tales maniobras generaron más de 40.000 datos, que se procesaron estadísticamente con técnicas multivariadas a efectos de minimizar el efecto aditivo de probabilidad de intervención del azar (error alfa), propio de los métodos univariados que abarcan numerosas variables dependientes. Para el análisis de componentes principales se seleccionaron variables que habían obtenido diferencias significativas para los efectos tratamiento y/o tiempo en el análisis univariado de medidas repetidas, con las cuales se conformaron 4 supervariables ortogonales, susceptibles de ser afectadas por estrés, hiponutrición y ontogenia. Las ganancias de peso fueron de 79,9 kg (666 g/animal/día) en C y 61,6 kg (513 g/animal/día) en E. Las variaciones de cortisol, aldosterona, fructosamina, glucosa, sodio, potasio, leucocitos, gamma globulinas y enzimas, examinadas en el marco de

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la ontogenia, sugirieron inexistencia de estrés. En cambio, las disminuciones de indicadores nutricionales proteicos, glucídicos, lipídicos y minerales revelaron hiponutrición en E. Los componentes principales seleccionados indicaron que la mayor parte de la variancia total ocurrió por hiponutrición y ontogenia, pero no por estrés. Se impone la obtención de suplementos balanceados más idóneos para el destete precoz de los terneros.

Palabras clave: ternero cruza cebú, ganancia de peso, alteraciones bioquímicas, estrés, hiponutrición.

INTRODUCTION

Early weaning, originated in Texas in 1960 and introduced in Argentina in 1971 [19], is still subjected to research to improve calves performance [5, 24]. In a recent trial, it was demonstrated that this handling technique causes lower hay consumption and calf negative energy balance, and it was suggested that beginning of early weaning should delay until animals are able to consume 0.75 kg/day of concentrated foods [14].

In beef livestock extensive breeding system on native pasture, growth delay of early weaned calves, in relationship to their nursing cronies, is attributed to stress [16, 23]. Nowadays, this neuroendocrine syndrome can be demonstrated biochemically, due to the fact that nervous tension and hormonal changes rebound in a characteristic way on certain analytes from animal internal environment [7]. Stress also generates ethological alterations [29], and frequently it causes death of the calf by abomasal ulcer [15].

Advance of stress is conditioned to the resistance exercised by animal [8]. The heterosis of half-bred Zebu cattle would confer a high grade of resistance and adaptability to unfavorable conditions [18]; calves of this crossbred are not very sensitive to social changes, they remain scarce time in contact with their mothers, and they develop less agonistic actions than those exercised by European races [12].

Keeping in mind such characteristics, it was established the hypothesis that growth delay in early weaned calves from this cross-bred could be due to malnutrition, rather than stress. Commercial balanced foods would possess appropriate concentration of nutritional principles, but makers do not indicate their origin source. Frequently they include chicken fat and meat, bird and fish flour [2, 3], which digestion could be difficult in nursling calf, because its gastrointestinal enzymes are programmed to degrade simpler nutrients, as lactoproteins, tributyrine and lactose [7]. On the other hand, opinions about true nutritional requirements of early weaned calves are still divergent. Some authors postulate -for example- the use of high fiber rate [2] and others are in favor of a low fiber rate [22].

The objective of the study was to investigate, through hematological and biochemical indicators, if early weaned half-bred Zebu calves growth delay is due to stress or inadequate feeding, using multivariate statistics, which are able to establish convincingly the causal relationship in grouped experimental and control animals.

MATERIAL AND METHODS

Randomized prospective designs applicable for univariate statistics (analysis of variance [ANOVA] of repeated measures) and for multivariate analysis of principal components [28], were employed. Treatment (early weaning) was the independent variable, and liveweight and laboratory values were quantitative continuous dependent variables, whose normal distribution enabled the use of parametric statistics [26]. Some covariables were excluded or minimized by the design (post-prandial changes, circadian rhythm, sex); other (climate, state of pasture, initial liveweight) were submitted to statistical studies (assay effect). Experimental design was balanced with equal number of replicas for each treatment (n = 15 animals); such number was fixed applying a sample size estimation method [17], which is based on the precision required to evaluate the studied data.

Four successive annual assays with experimental (E, weaned calves) and control (C, nurslings calves) lots, which comprised four months each (from late November or early December, until late March or early April), were carried out. A hundred and twenty animals integrated the total randomized group (60 experimental and 60 control), which were distributed in a number of 30 calves per year (15 replicas for each treatment).

Experimental subjects were half-bred Zebu x British nurslings calves from 60-75 days old and 60-90 kg liveweight, 50% females and 50% males. The last had been castrated few days after birth (reduction of sex effects). Calves had homogeneous phenotypical characteristics, and were clinically healthy, deparasitized and vaccinated according to habitual sanitary handling of the farm, which was located in northwest of Corrientes Province (Argentina). This zone is characterized by subtropical climate (annual mean temperature: 21ºC, ranges from 42 to -2ºC), with one or two annual frosts, and rains from 1,200-1,300 mm/year [13]. Pastures are mainly perennial gramineous of summery cycle, and they has approximately 6% of crude protein (1% digestible), 2-3% of fat, 30-40% of non-nitrogen extract, 25-35% of cellulose, and 10% of ash [21]. Extensive breeding is the main cattle activity. Zonal production is upper to 300,000 calves/year [2], with a weaning liveweight (8^{th} month) nearly to 180 kg [20].

In November-December of each year, lots C and E were randomly integrated. Identified by tags, both C and E lots stayed in contiguous plots with similar soil

and pasture (native grasses). Calves from lot C, appointed to conventional weaning (to be carried out in March-April), continued feeding with maternal milk and pasture, as long as those from lot E were submitted to early weaning and fed on pasture, supplemented with a balanced food. Supplement contained crude protein (16%), crude fiber (7%), ether extract (4%), Ca (0.64%), and P (0.53%), with ME = 2.77 Mcal/kg (DM), and it was initially supplied in a proportion of 1.5% LW. This percentage gradually decreased in function of progressive increase of pasture intake (final = 0.7% LW).

In both C and E lots, the take of samples began at day 0 (moment of early weaning) and continued during days 7, 14, 21, 28, 60, 90 and 120. Highest initial frequency (weekly) in relation to final one (monthly), was planned foreseeing that most important changes could happen nearby to the shock of early weaning. Continuation of sampling until the fourth month of assay (six months of calves age) was projected to verify differences among values of nutritional-metabolic analytes from both C and E lots, at the moment of conventional weaning.

In morning hours (8-9 am: exclusion of circadian rhythm), with animals under basal condition (fast: exclusion of post-prandial changes), individual weighings and blood extractions by jugular venepuncture, were made. A blood aliquot was treated with anticoagulant (EDTA); the other was appointed to obtain serum, materials that stayed refrigerated (5ºC) until its analytic prosecution, carried out before 6 hours after extraction. Daily, behavior and health state from all animals, as well as the rate of supplement consumption, were verified with help of the farm staff.

Hematological and biochemical tests were planned to detect stress, as well as to verify the nutritional and metabolic state from animals. Using conventional laboratory techniques [1] and Wiener, Randox, Biopur, DPC-Lab and Boehringer reagents, serum concentration were determined for cortisol (chemiluminiscence enzyme immunoassay), aldosterone (radio-immune assay by competitive technique), fructosamine (nitroblue tetrazolium), glucose (oxydase-peroxydase), triglycerides (lipase-peroxidase), total cholesterol (oxidase-peroxidase), cholesterol bound to high and low density lipoproteins, HDL-C and LDL-C (precipitation of selective lipoprotein and enzymatic determination of cholesterol), alpha and beta lipoproteins (electrophoresis on agarose gel and densitometry), total protein (biuret), albumin and alpha, beta and gamma globulins (electrophoresis on cellulose acetate and densitometry), urea (urease), alkaline phosphatase (ALP, phenylphosphate), gammaglutamyl transferase (GGT, p-nitroanilide), creatine phosphokinase (CPK, creatine-ATP), lactate dehydrogenase (LDH, dinitrophenylhydrazine), aspartate aminotransferase (AST, aspartate-ketoglutarate), copper (bathocuproine), magnesium (calmagite), calcium (cresolphtaleincomplexone), inorganic phosphorus (phosphomolybdate), iron (PBTS), and chloride (mercuric tiocianate). Sodium and potassium were evaluated by flame photometry. Quality control of biochemical determination were carried out by liophilized *ad-hoc* patterns.

Erythrocytes, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), concentration of MCH (CMCH), and total leukocytes were determined in an hematological electronic analyzer adapted for calf blood [6]. Absolute leukocytary formula was obtained by microscopy, through differential recount of 200 white blood cells from smears stained by Giemsa.

Keeping in mind that in 8 sequential opportunities, 42 dependent variables were determined on 120 animals, a total of 40,000 data were generated; such magnitude contributed to increase the statistical certainty [27]. Helped by the software "Statistica", distributive normality was verified by Wilk-Shapiro (WS) method, and values initial homogeneity was checked by overlapping of confidence intervals (CI±95%). Parametric descriptive statistics included measures of central tendency (arithmetic mean, $\bar{x}$) and dispersion (standard deviation, SD). Homogeneity of the variance was estimated by Bartlett test. ANOVA of repeated measures was included to know the statistical significance of treatment effect (early versus conventional weaning) and time effect (growth, ontogeny), as well as the interaction between them. Significance of differences between C and E for each sampling date, were determined by a mean comparison test (Tukey HSD).

The effect of assay (difference attributed to year of trial, climate, pastures), was evaluated through the interaction treatment x assay x time; the same criterion was used for the effect of sex. These data are not included because they resulted not significant. Degree of linear association was established by Pearson test. Significant correlations were grouped, for their best interpretation, according to the type of information gave by each variable, since the screening included indicators of ontogeny, stress and nutritional state (metabolism). Many of these indicators are susceptible to be modified at the same time by several causes, as it is detailed later on. For all inferences, an alpha risk of 5% was specified, below which the equality null hypothesis was rejected, except in multivariate analysis, where rigorousness increased to 1%.

The extraction of "principal components", which are axes of coordinates that inform the connection (gradients) among dependent variables, were included to point out the most important guidelines to explain the total variation, allowing an overall interpretation from the voluminous group of obtained data. These multivariate statistics allowed to reduce extensive dimensions toward some few orthogonal variables ("supervariables"), independent to each other (they did not overlap their variance). Such orthogonal variables (factors) constituted linear combination of dependent variables; some of them contributed with bigger "load" than other, to define the meaning of each factor (component) [28].

To calculate multivariate ANOVA, 23 variables (with 120 repetitions each one, in 8 different times) were selected, starting from those that had obtained significant differences for the effects of treatment and/or time in univariate ANOVA. Selection included variables able of being affected in a different way by the phenomena involved here, such as:

1. Variables preponderantly susceptible to nutritional state and ontogeny, rather than stress (liveweight, total protein, albumin, urea, HDL-C, Cu, P, Fe, MCV).

2. Variables primarily susceptible to stress and ontogeny, rather than nutritional state (cortisol, aldosterone glucose, fructosamine, total leukocytes, neutrophils, lymphocytes, eosinophils).

3. Variables equally susceptible to changes by ontogeny, stress, and nutritional state (triglycerides, cholesterol, erythrocytes, hematocrit, hemoglobin, and ALP).

Eigenvalues (own values), scorer factors, and loading factors, were obtained by factorial analysis. A 3 way ANOVA was made: between (1-treatment, 2-assay) and within (3-time), including interactions (1x2, 1x3, 2x3 and 1x2x3) for each factor or component. Ulteriorly, mean comparison tests (treatment x time) were carried out using the Tukey HSD method, decreasing the risk to p = 0.01, coefficient that arises when conventional risk (0.05) is divided by 4, number of considered factors, according to Bonferroni correction [27].

By means of correlated matrixes algebra, applied technique generated a system of coordinates axes (principal components, supervariables) which magnitude was directly proportional to the variance percentage that they were able to explain, informing the variation gradient of the group of data, that is to say: which were the most important variables to explain the total variance. Secondary axes explained the additional variation, as long as a part of the variance remained as not explained, being attributable to not detected concomitant variables (covariables). These tests allowed to infer the causality (causal relationship) of the studied phenomenon [28].

RESULTS AND DISCUSSION

Initial laboratory values framed in the reference interval for the geographical area, breed, and age of animals [7]. Balanced supplement was totally consumed; rejections by bad palatability were not registered. Clinical alterations were not detected, except a state of uneasiness in early weaned calves, characterized by an increase of the frequency of vocalizations (bleats) during the first 24 hours. Short duration of this behavioural anomaly would reject the probability that these calves have suffered stress, because in this syndrome the bleats continue during several weeks and are accompanied by other ethological, postural and ambulatory changes [29].

Initial and final values of analytes determined in C and E by univariate ANOVA of repeated measures, are shown in Tables 1 and 2. Decrease of total protein, albumin, urea, hemoglobin, copper, iron, magnesium, globulin gamma, HDL-C and lipoprotein alpha in E could be attributed to malnutrition [25]. In grouped C and E animals, increases of cortisol, CPK, hematocrit, MCV, hemoglobin, MCH, CMCH, eosinophils, and urea, as well as decreases of aldosterone, ALP, and total leukocytes, could be imputed to ontogeny [1]. Decreases of glucose, fructosamine, triglycerides, total cholesterol, inorganic phosphorus and erythrocytes, could be due as much for ontogeny as for malnutrition [7, 25]. On the other hand, if stress had been present, it would have caused decreases of potassium, lymphocytes, eosinophils, and globulin gamma, as well as increases of cortisol, glucose, fructosamine, aldosterone, and sodium [8].

Results obtained by univariate techniques are not discussed detailedly, because they have been treated in previous publications [9, 10, 11]. However, it is important to emphasize that early weaned lots registered lower weight gains than control lots (Figure 1). For total nurslings calves, mean liveweight gain was 79.9 kg (666 g/animal/day), as long as the weaned calves gained only 61.6 kg (513 g/animal/day). Considering the year of trial as covariable, interaction assay x treatment x time was not significant (p = 0.40). Growth delay verified in E coincides with results obtained in other investigations performed in northeastern argentine, where daily liveweight gains were always lower in early weaned animals: 548 versus 707 g [22], 452 versus 663 g [30], and 516 versus 630 [3]. Final difference between C and E was 19.3 kg, almost identical to that registered by others (20 kg) [4].

Results of univariate statistics are eloquent about the malnutrition state and the nonexistence of a biochemically demonstrable stress in E, but they suffer from the additive effect from probabilities of random intervention. It occur when dependent variables are numerous. In this way, values of probability are superior than the nominal ones (0.05), because for each performed statistical test, increase the chances of making type I (alpha) error, for which a true null hypothesis can be rejected [27]. For this reason, multivariate techniques (principal components) were performed; they allowed to interpret the numerous obtained data in a combined way [28].

The first four principal components were retained, applying the factorial analysis *scree plot* criterion. The first eigenvalue was 6.2 (variance associated to principal component 1); it explained 27.2% of total variance. The second (4.6) explained 19.9%, the third (2.1) explained 9.0% and the fourth (1.3) explained 5.7% of total variance (Table 3). These four factors were able to explain 61.9% of the total variance.

The loading of each variable, for each one of retained principal components, are detailed in Table 4. Underlined values were those whose relative contribution was important inside each factor, reason for which they were used in statistical analysis. Factor 1 presented strong negative correlation with liveweight, total protein and urea (variables that decrease by malnutrition), and positive correlation with total leukocytes, lymphocytes,

Table 1. Values of hormones, carbohydrates, lipids, proteins and enzymes obtained in C and E ($\bar{x} \pm SD$) by means of univariate statistics (repeated measures ANOVA).

variable	initial (day 0)		final (day 120)		effects	
	C (n = 60)	E (n = 60)	C (n = 60)	E (n = 60)	Ti	Tr
cortisol (ug/dl)	2.2±0.5 a	2.4±0.6 a	3.4±0.8 b	3.7±0.9 b	↑S	NS
aldosterone (pg/ml)	348±12 a	351±13 a	288±11 b	291±14 b	↓S	NS
glucose (g/l)	1.52±0.33 a	1.42±0.35 a	0.94±0.15 b	0.88±0.13 b	↓S	NS
fructosamine (umol/l)	297±35 a	299±39 a	226±33 b	221±32 b	↓S	NS
triglycerides (g/l)	0.42±0.13 a	0.43±0.12 a	0.36±0.10 b	0.21±0.09 c	↓S	↓S
total cholesterol (g/l)	1.15±0.32 a	1.09±0.31 a	1.07±0.29 a	0.94±0.25 b	↓S	↓S
HDL-C (g/l)	0.78±0.13 a	0.81±0.14 a	0.70±0.12 b	0.66±0.11 b	↓S	↓S
LDL-C (g/l)	0.21±0.09 a	0.17±0.08 a	0.24±0.08 b	0.23±0.13 b	↑S	NS
lipoprotein alpha (%)	83.7±6.6 a	85.2±5.7 a	82.9±6.3 a	78.4±5.5 b	↓S	↓S
lipoprotein beta (%)	16.3±6.4 a	14.8±5.7 a	17.1±5.9 b	20.5±5.0 c	↑S	↑S
total protein (g/dl)	5.73±0.34 a	5.77±0.29 a	6.91±0.38 b	6.04±0.31 c	↑S	↑S
albumin (g/dl)	3.29±0.28 a	3.31±0.26 a	3.39±0.29 a	3.20±0.24 b	NS	↓S
globulin alpha (g/dl)	0.75±0.13 a	0.78±0.14 a	0.79±0.13 a	0.78±0.13 a	NS	NS
globulin beta (g/dl)	0.79±0.16 a	0.78±0.14 a	0.82±0.11 b	0.82±0.13 b	↑S	NS
globulin gamma (g/dl)	0.88±0.13 a	0.89±0.16 a	1.91±0.28 b	1.24±0.27 c	↑S	↑S
albumin/globulin ratio	1.38±0.16 a	1.37±0.15 a	0.94±0.16 b	1.13±0.17 c	↓S	↓S
activity of ALP (UI/l)	453±51 a	459±40 a	331±30 b	325±32 b	↓S	NS
activity of AST (UI/l)	31.8±7.8 a	32.3±6.6 a	30.6±8.1 a	30.3±7.1 a	↓S	NS
activity of LDH (UI/l)	597±127 a	571±131 a	584±126 a	592±122 a	NS	NS
activity of GGT (UI/l)	14.8±6.9 a	15.7±7.2 a	14.6±6.6 a	14.9±6.8 a	NS	NS
activity of CPK (UI/l)	111±28 a	114±27 a	170±33 b	163±38 b	↑S	NS

Ti: time, Tr: treatment, S: significant, NS: not significant. Arrows indicate the sense of the variations verified in E (Tr) or in both lots (Ti). In each line, different letters indicate significant differences among means (Tukey). Differences between C and E began to be significant between days 7 and 14, except for total protein (day 28).

aldosterone, glucose, and ALP (variables that decrease by ontogeny).

High load variables correlated positively to Factor 2 were MCV, neutrophils, and eosinophils (variables that increase by ontogeny). High load variables correlated negatively to Factor 2 were hematocrit, erythrocytes, hemoglobin, albumin, Fe, P and Cu (variables that decrease by malnutrition). The most important positive load for Factor 3 corresponded to cholesterol and triglycerides, lipids whose serum concentration decrease by malnutrition. Factor 4 revealed a marked negative correlation to neutrophils and erythrocytes, as well as a positive correlation to MCV and fructosamine; this variability is attributed to random, because it belongs to the axis of lower load, where it could be hidden the variation attributable to assay (year). Variables of higher load registered scarce overlapping rate, indicating that each axis was expressing groups of variables different to each other, with acceptable discrimination.

ANOVA of repeated measures registered highly significant differences for treatment (1), assay (2) and time (3), as well as for the interactions treatment x time (1x3), assay x time (2x3) and assay x treatment x time (1x2x3), for all considered factors (Table 5). Interaction treatment x assay (1x2) was not significant. Statistical significance of interaction 1x3 enabled the ulterior verification of variations happened by effect of the treatment, in each one of the times of sampling. Test of mean comparison (Tuckey HSD, alpha = 0.01) indicated that first interaction (day 0) was not significant for any factor, but from second taking of samples (day 7) until the last one (day 120), all interactions were highly significant, except in day 21 for Factor 4.

Bidimensional representation of displacement gradients from C and E lots, is shown in Figure 2, which was obtained by the *scatterplot* technique of the used software, through the axes determined by Factor 1 and Factor 2. These factors represent supervariables of grouped dependent variables that explain almost 50% of total variance. Contours represent displacements registered by lots C (n = 60) and E (n = 60). Starting from a centroid (intersection of zeros from absis and ordinate relative scales), Factor 1 defines two areas (negative left and positive right), and Factor 2 demarcates other two areas (positive superior and negative inferior). The overlapping of factors generates four areas: right superior (positive for both factors), left inferior (negative for both factors), left superior (negative for Factor 1 and positive for Factor 2) and right inferior (positive for Factor 1 and negative for Factor 2).

According to the loading of obtained principal components (Table 4), it should be interpreted that right to left displacements through axis 1 (vertical) are directly proportional to time advance (ontogeny), and in smaller extent it reflects increase of nutritional indicators. On the other hand, axis 2 (horizontal) responds primarily to nutritional factors (higher values in inferior area, and lower values in superior area), but scarcely to ontogeny. It is derived from fact that positive correlation is manifested in sharp angles, and negative correlation in obtuse angles, regarding each axis.

Table 2. Values of minerals, trace-elements, erythrogram, leukogram, urea and liveweight obtained in C and E ($\bar{x} \pm SD$) by means of univariate statistics (repeated measures ANOVA).

variable	initial (day 0)		final (day 120)		effects	
	C (n = 60)	E (n = 60)	C (n = 60)	E (n = 60)	Ti	Tr
sodium (meq/l)	144±5 a	142±6 a	142±5 a	143±6 a	▯S	NS
potassium (meq/l)	4.53±0.46 a	4.51±0.49 a	4.50±0.49 a	4.56±0.51 a	NS	NS
chloride (meq/l)	95.8±6.8 a	96.2±6.3 a	96.0±7.4 a	95.7±5.9 a	NS	NS
calcium (mg/dl)	9.08±0.79 a	9.25±0.83 a	9.20±0.91 a	9.28±0.96 a	NS	NS
magnesium (mg/dl)	2.47±0.32 a	2.48±0.34 a	2.56±0.37 b	2.43±0.36 c	NS	▯S
in.phosphorus (mg/dl)	9.6±1.1 a	9.8±1.0 a	7.9±1.0 b	6.8±1.1 c	▯S	▯S
iron (ug/dl)	109±17 a	113±20 a	112±17 a	92±20 b	▯S	▯S
copper (ug/dl)	82±18 a	78±21 a	80±19 a	59±20 b	▯S	▯S
erythrocytes (T/l)	8.97±0.93 a	8.72±0.89 a	8.33±0.97 b	7.60±0.99 c	▯S	▯S
hematocrit (%)	38.3±2.8 a	39.1±2.9 a	40.3±3.0 b	36.1±3.1 c	▯S	▯S
MCV (fl)	42.7±4.3 a	44.8±4.0 a	48.4±4.3 b	50.9±4.6 b	▯S	NS
hemoglobin (g/dl)	12.1±1.3 a	12.4±1.2 a	13.8±1.1 b	11.7±1.4 c	▯S	▯S
MCH (pg)	13.8±1.1 a	13.3±1.0 a	16.1±0.9 b	15.1±1.0 b	▯S	▯S
CMCH (%)	31.6±1.8 a	31.7±1.7 a	34.2±1.7 b	30.7±2.5 a	▯S	▯S
total leukocytes (G/l)	14.31±1.69 a	14.19±1.59 a	9.76±0.90 b	12.08±1.08 c	▯S	▯S
neutrophils (G/l)	3.58±0.57 a	3.67±0.62 a	3.78±0.59 b	4.12±0.59 c	▯S	▯S
lymphocytes (G/l)	10.22±1.45 a	10.01±1.31 a	5.9±0.76 b	7.26±0.95 c	▯S	▯S
monocytes (G/l)	0.44±0.08 a	0.43±0.07 a	0.31±0.09 b	0.36±0.09 b	▯S	NS
eosinophils (G/l)	0.07±0.05 a	0.08±0.06 a	0.36±0.19 b	0.33±0.15 b	▯S	NS
urea (g/l)	0.22±0.03 a	0.23±0.03 a	0.30±0.04 b	0.25±0.03 a	▯S	▯S
liveweight (kg)	78.9±6.9 a	77.8±7.0 a	158.7±11.7 b	139.4±11.6 c	▯S	▯S

Ti: time, Tr: treatment, S: significant, NS: not significant. Arrows indicate the sense of the variations verified in E (Tr) or in both lots (Ti). In each line, different letters indicate significant differences among means (Tukey). Differences between C and E began to be significant between days 7 and 14, except for copper (day 21) and lymphocytes (day 28). Basophils were not included because their concentration was very scarce.

The dispersion diagram indicates that in the beginning of assays (day 0), control and experimental animals shared common areas of right inferior quadrant (90% overlapping: homogeneity). When advancing the time, control animals moved toward the left (ontogeny), without abandoning the inferior quadrant (high nutritional values). Experimental calves moved toward the superior quadrant (low nutritional values), and then toward the left, to finish (day 120) in an area different to that of control lot (10% overlapping), retarded in the axis of ontogeny.

Interpretation of registered gradients allows to extract similar conclusions to those indicated for univariate analysis, reaffirming that early weaned calves are different to nursling calves for nutritional reasons, but not for stress. Excluding the ontogeny effects (that impacted in both lots), statistics from lot E demonstrated that stress indicators (cortisol, aldosterone, glucose, fructosamine, leukocytes) were not altered, meanwhile nutritional indicators were decreased. Scatterplots revealed that lower liveweight gain of E moved toward the same direction to that corresponding to lower values of protein, albumin, urea, triglycerides, Cu, P, Fe, hemoglobin, erythrocytes and hematocrit.

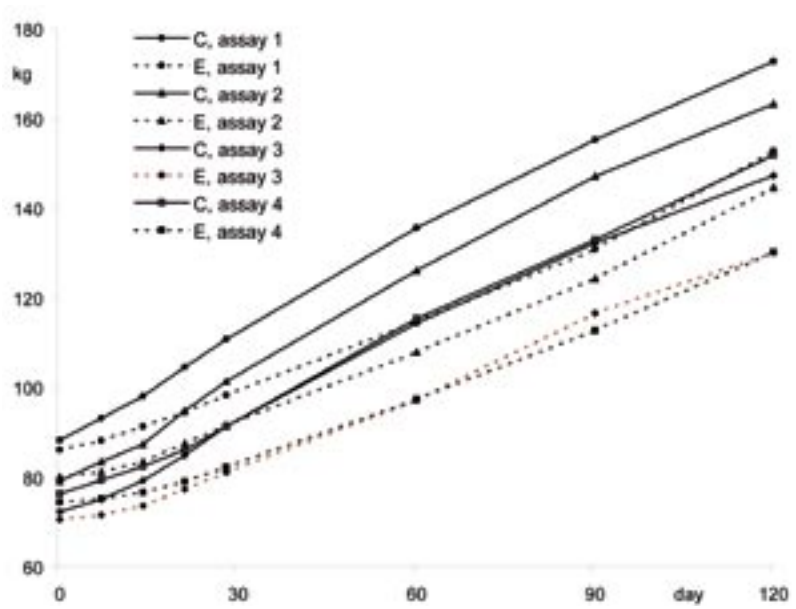

Figura 1. Evolution of liveweight from control and experimental calves, in each assay (year).

Figure 3 point out that malnutrition, ontogeny, and in much smaller extension the differences among assays, explained 62% of total variance, as long as stress indicators had not participation in such explanation. Coincidently, Zebu x British early weaned calves registered lower liveweight gain than those submitted to conventional weaning, but they did not show

Table 3. Variance explained by each obtained eigenvalue.

factor	eigenvalue	% of explained variance	accumulated *eigenvalues*	accumulated explained variance (%)
1	6.259805	27.21654	6.25981	27.21654
2	4.578315	19.90572	10.83812	47.12226
3	2.083494	9.05867	12.92161	56.18093
4	1.314410	5.71483	14.23602	61.89576

Table 4. Correlation ("loading") of the variables for each factor.

variable	factor 1	factor 2	factor 3	factor 4
albumin	-0.334650	-0.626472	-0.424017	0.188814
total cholesterol	0.191631	-0.082581	0.711348*	0.301682
copper	-0.057774	-0.593973	0.043025	-0.279913
iron	-0.187532	-0.719339*	0.176272	0.133855
erythrocytes	-0.124607	-0.684148	-0.003605	-0.347471
hemoglobin	-0.408279	-0.742790*	0.006744	-0.047945
hematocrit	-0.254176	-0.688839	0.137765	0.008496
liveweight	-0.863735*	0.131390	0.277962	-0.164369
inorganic posphorus	0.302147	-0.700385*	0.229365	-0.128132
total protein	-0.736228*	-0.289341	-0.132850	0.253391
triglycerides	0.157122	-0.414168	0.535492	-0.071373
urea	-0.628443	-0.172654	0.357603	0.108436
MCV	-0.066181	0.313752	0.348354	0.399984
eosinophils	-0.595854	0.315769	0.180728	0.102608
HDL-cholesterol	0.267883	-0.473473	0.257737	0.313581
total leukocytes	0.838593*	0.178174	0.069374	-0.070136
lymphocytes	0.889354*	-0.006282	-0.063700	0.036474
neutrophils	0.162151	0.308798	0.482366	-0.587106
aldosterone	0.735398*	-0.425429	-0.145231	-0.004581
activity of ALP	0.773116*	-0.315962	0.208922	-0.076324
cortisol	-0.518367	0.268696	0.328773	0.090530
glucose	0.668137	0.178547	0.315119	0.258280
fructosamine	0.470614	-0.295378	-0.222385	0.378596
explained variance	6.26	4.58	2.08	1.31
total participation	0.27	0.20	0.09	0.06

*Significant. In each column, numbers underlined indicate the values taken into account to establish the injerence of each factor according to their highest relative contribution ("loading").

Table 5. Analysis of variance for repeated measures (Anova-3 way), days 0 to 120.

effect	Factor 1	Factor 2	Factor 3	Factor 4
treatment (1)	HS	HS	HS	HS
assay (2)	HS	HS	HS	HS
time (3)	HS	HS	HS	HS
interaction 1 x 2	0.04	0.87	0.41	0.46
interaction 2 x 3	HS	HS	HS	HS
interaction 1x2x3	HS	HS	HS	HS
interaction 1 x 3	HS	HS	HS	HS
day 0 (1 x 3)	0.14	0.06	0.92	0.42
day 7 (1 x 3)	HS	HS	HS	HS
day 14 (1 x 3)	HS	HS	HS	HS
day 21 (1 x 3)	HS	HS	HS	0.99
day 28 (1 x 3)	HS	HS	HS	HS
day 60 (1 x 3)	HS	HS	HS	HS
day 90 (1 x 3)	HS	HS	HS	HS
day 120 (1 x 3)	HS	HS	HS	HS

HS: highly significant ($p < 0.001$)

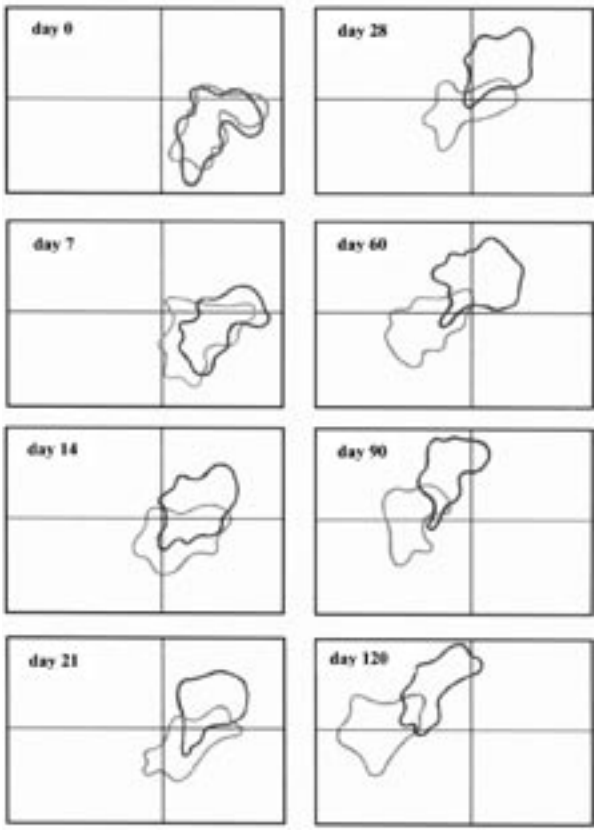

Figura 2. Displacement gradients of control (gray outline) and experimental groups (black outline). The separation between both lots was significant starting from day 7. Displacements along vertical axis (Factor 1) represent ontogenic changes (growth), and those of horizontal axis (Factor 2) are due to modifications from nutritional indicators (high values in inferior quadrants, and low values in superior quadrants).

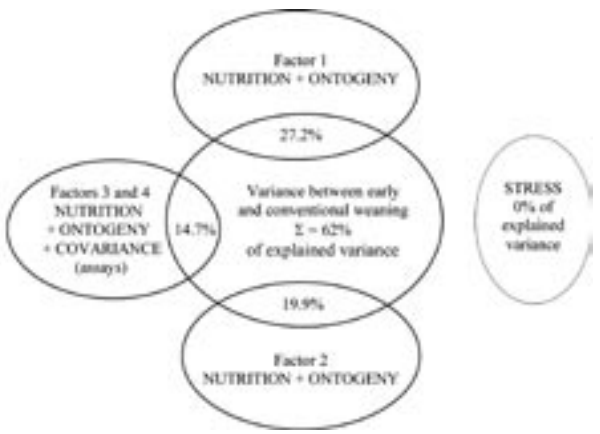

Figura 3. Participation of each factor in the explanation of total variance.

increase of acute phase proteins (stress indicators), as haptoglobin and ceruloplasmin [5] .

In conclusion, early weaning does not cause a biochemically demonstrable stress in half-bred Zebu calves, although it generates a malnutrition state characterized by blood nutritional indicators concentration lower than those registered on nursling calves. To make more profitable the practice of early weaning it is necessary to obtain more efficient balanced supplements, whose cost does not alter the economic equation of productive system.

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