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Abstract

Public concern for animal welfare has progressively grown over the recent years. In this context, stress has a great economical impact on growth of animals and quality of animal products. The development and validation of methods to assess animal stress, particularly at the farm level, is desirable to evaluate animal production systems. Piemontese breed is traditionally tie-stall housed in the fattening period. Hence, the objective of this study was to characterize a profile of physiological and haematological changes of Piemontese beef cattle under different management conditions (tie-stall and loose housing). Our results suggest that the housing system is an important factor in animal welfare. Indeed, the values of the total protein, lysozyme, cortisol, serum and faecal corticosterone concentration and GR-α gene expression indicate that the tie-stall housing is more stressful than the loose system. All the alterations highlighted in this study considered together may be effective biomarkers of stress and disease susceptibility.

Keywords: beef cattle; housing; welfare; immunology, physiology

1. Introduction

Piemontese cattle is one of the most important Italian breeds for meat production, characterized by muscular hypertrophy, better known as the *double-muscled* phenotype. The rearing and fattening period of the Piemontese young bulls is traditionally carried out in tie-stall, also to satisfy the dealers' request. Due to the need of optimizing the production processes and to meet the consumers' expectation, in recent years the researchers interest on the animal welfare is increased. Several authors focused their attention on the effects of husbandry conditions on behavioural, biochemical, functional, endocrine and pathological

variables (Fike and Spire, 2006, Broom, 2007; Gupta et al., 2007). In this context, the Scientific Committee on Animal Health and Animal Welfare (SCAHAW) discourages tie-stall and favours a loose housing system. The EU, by means of several laws, aims to maximize livestock production, ensuring at the same time appropriate welfare standards to animals. The best scientific method to assess animal welfare is to measure the response of animals on the basis of certain functional indicators, which have been classified by Smidt (1983) into 4 criteria groups: behavioural, physiological, pathological and performance. Particularly, among physiological measures, neuro-endocrine hormones are often evaluated. In details, the release of adrenal hormones (e.g cortisol) is an important physiological mechanism indicating the animal adaptation to some stressors. Stress is a physiological response to several exogenous or endogenous stimuli that normally cause neuro-endocrine activation and response of the endocrine system, mainly characterized by the concomitant release of catecholamines and glucocorticoids (GCs) from the adrenal glands (Rushen et al., 2008). In addition, high stress levels are able to reduce immune system response efficiency, then promoting diseases onset. Diseases could obviously entail economic losses due to veterinary costs, poor growth performance, culling and early deaths. Other physiological variables that can be considered are the heart activity, body temperature and glucocorticoid receptor (GR) expression (von Borell et al., 2007; Odore et al., 2011). There are many potential sources of stress that could affect animals during the fattening period, especially within the intensive and industrial farms, the space allowance, space at the manger, flooring, micro-climate and human-animal relationship represent some of these welfare risk factors. According to several authors (Leaver, 1999; Weary and Taszkun, 2000; Fregonesi and Leaver, 2001), the housing system is a factor that strongly affects the welfare quality, especially in regard to health condition and behaviour expression. Traditional

rearing techniques often ensure the final product quality requested by the consumer, however in so doing, animal behaviour and welfare may be compromised (Xiccato et al., 2002). The general aim of this work was to enhance the knowledge of Piemontese beef cattle fattening system, in order to better understand how different housing methods (tie-stall and loose housing) could act as stress factor. The study of physiological and haematological profiles could help to identify potential biomarkers of stress useful in future to guarantee both animal welfare and farmer‘s economic advantage and sustainability. A trial was carried out to evaluate the influence of two rearing systems, in tie-stall and in loose housing, on physiological and haematological parameters in beef cattle.

2. Materials and methods

2.1. Animals and experimental design

Twelve healthy male, Piemontese beef cattle, were monitored in this trial. None of the animals were castrated. All animals were kept in the same condition (loose housing), during the acclimation period (1 month) before the first sampling (day 0, d0). The cattle were randomly assigned as follows: a) six cattle (11.07 ± 0.73 months) (mean age ± SEM) were housed in loose conditions in which each animal had a pen area allowance of 5.0 m^2; b) six cattle (11.67 ± 0.49 months) (mean ± SEM) were housed in tie-stall conditions in which cattle were tied by a tether fixed to the feed trough (space allowance 2.00 x 1.00 m/head). All animals were accommodated on a solid concrete floor bedded with straw. All animals were fed a concentrated diet of corn silage, corn, hay and a commercial protein supplement. Water was supplied *ad libitum*.

2.2. Samples collection and laboratory investigation

All samples were collected monthly for four times (d 0, d 30, d 60 and d 90 from the beginning of trial). Blood samples were aseptically collected from each animal via direct jugular venipuncture between 8:30 and 10:30 a.m. into the Vacutainer® tubes (BD, Franklin Lakes, NJ, USA) containing ethylene diaminotetraacetic acid (EDTA) for haematology determination, without additive for hormonal and biochemical parameter concentration measurements, containing sodium citrate for fibrinogen evaluation and acid-citrate-dextrose (ACD) for peripheral blood mononuclear cells (PBMCs) separation. Blood samples were maintained at 2°C until they arrived at the laboratory.

After blood sampling, fresh faeces were collected from the rectal ampulla.

The following physiological indicators were analyzed:

- Haematological: white blood cells (WBC) number, lymphocyte number and neutrophil number using an automated haematology analyzer (ADVIA® 120, Siemens, Munich, Germany).
- Biochemical: total protein, fibrinogen, albumin, globulin, lysozyme and superoxide dismutase (SOD) activity. The total protein and albumin concentrations were measured using an automatic analyzer (ILAB300, Instrumentation Laboratories, Milan, Italy). The plasma was assayed for fibrinogen using the Clauss method and the Fibrinogen Reagent (Roche, Basel, Switzerland) on an automated analyser (Start® 4, Diagnostica Stago, Asnières sur Seine, France). The globulin and albumin to determine the albumin/globulin ratio (A/G) were measured through serum protein electrophoresis using the Hydragel kit (Sebia, Lisses, France) on Hydrasys instrument (Sebia). The lysozyme inhibition zone assay was performed as follows: agarose (1%; Sigma, St. Louis, MO, USA) was prepared in a 67 mM sodium phosphate buffer, pH 6.3, containing 5 mg/100 mL lyophilised Micrococcus

lysodeikticus (ATCC N. 4698) (Sigma). The melted agarose (7 mL) was spread in 3.5 cm Petri dishes. After solidification (30 min), wells with a 2 mm diameter were made in the agarose, and 10 µL of undiluted sample was placed in each well. Each plate was incubated at 37°C for 16 h. The lysozyme concentration was proportional to the diameter of the inhibition zone, and the concentration was quantified using a standard curve generated with lysozyme from chicken egg white (Fluka, St. Louis, MO, USA) prepared in 67 mM sodium phosphate buffer. Samples and standards were assayed in duplicate wells. The SOD activity was evaluated with the commercial kit by Cayman Chemical Company (Ann Arbor, MI, USA) according to the manufacturer's instructions.

- Hormonal: serum cortisol and corticosterone and faecal corticosterone. The concentrations were evaluated with the commercial kits by Arbor Assay (Ann Arbor, MI, USA) according to the manufacturer's instructions. The colorimetric reading was carried out using a spectrophotometer at 450 nm.

- Gene expression analysis: the isolation of the PBMCs was performed by a density gradient centrifugation method. Briefly, 15 mL of blood were carefully layered onto an equal volume of Ficoll-Paque™ (GE Healthcare, Chalfont St. Giles, UK) and centrifuged at 750 x g for 30 min at 20°C. The PBMCs were carefully collected from the central opaque interface without disturbing the other layers and transferred to a clean tube and additioned by two volumes of RPMI-1640 (Sigma). The samples were centrifuged at 996 x g for 15 min at 20°C. The supernatant was discarded, and the pellet of PBMCs was resuspended in 1.5 mL of QIAazol® Lysis Reagent (Qiagen, Hilden, Germany), divided into two aliquots and stored at –80°C until RNA extraction. The PBMCs were disrupted using a TissueLyser II (Qiagen) with stainless steel beads; the total RNA extraction was performed following the manufacturer's protocol. RNA concentration and purity were determined by spectrophotometry, and RNA

integrity was evaluated using an automated electrophoresis station (Experion™ Instrument, Bio-Rad, Hercules, CA, USA). The cDNA was synthesised from 1 µg of total RNA using the QuantiTect® Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. To determine the relative amount of glucocorticoid receptor α (GR-α) transcript, the cDNA was subjected to quantitative polymerase chain reaction (qPCR) using the IQ™5 detection system (Bio-Rad) and respective gene primers in an IQ™ SYBR® Green Supermix (Bio-Rad) with the following settings: 3 min at 95°C and 40 amplification cycles (each 10 sec at 95°C, 30 sec annealing at 60°C). The primer sequences were designed using Primer Express® (version 1.5; Applied Biosystems, Foster City, CA, USA) (Table 1). The cyclophilin A (PPIA) gene was used as a housekeeping gene control, as reported in previous literature (De Maria et al., 2010). The amount of relative gene expression was calculated using a relative quantification assay according to the comparative Ct method (ΔΔCt method) when the primer efficiencies were similar. Before each qPCR, we performed a validation experiment to demonstrate that the amplification efficiencies of the target and reference genes were approximately equal. The comparative Ct method is an abbreviated version of the relative standard curve method. When the difference between the primer efficiencies of the target and the housekeeping genes was too large, the relative standard curve method was applied, and the expression abundance of each gene was corrected for its efficiency (Wong and Medrano, 2005).

The amount of relative gene expression was calculated using a calibrator. The calibrator is one of the experimental samples and is necessary when the samples are not amplified in the same PCR run (Wong and Medrano, 2005). The sample used as the calibrator was the same for all runs. Each of the normalised target values was divided by the calibrator normalised target value to generate the relative expression

abundances. The calibrator is the 1x sample, and all other quantities are expressed as an n-fold difference relative to the calibrator (Wong and Medrano, 2005). The calibrator was retrotranscripted in a large enough quantity to complete all runs and stored at –20°C in single use aliquots. Every sample was tested in duplicate, and the results are presented as the least squares means.

2.3. Statistical analysis

All statistical analysis were performed using IBM SPSS Statistics version 19. Data were analysed using the MIXED linear model procedure of SPSS. Animal was the experimental unit and was specified as a repeated measures effect, and the dependence within animal was modelled using an unstructured covariance structure. The variables were tested for normality using the Kolgomorov–Smirnov test. The data that were not normally distributed (lysozyme and GR-α) were log transformed. The effects of time, housing model and time x housing model interaction were listed in the model statement. Least squares means were estimated and differences were tested using the pairwise comparison (t-test) with Bonferroni adjustment for multiple comparisons. A probability of $P < 0.05$ was selected as the level of significance.

3. Results

3.1. Haematological variables

There was an effect of time ($P < 0.05$) and time x housing model interaction ($P < 0.05$) for WBC number which significantly increased on d 90 (Table 2; Fig. 1A). No factor or interaction influenced the neutrophil and lymphocyte number, and the N/L ratio (Table 2).

3.2. Biochemical variables

The total protein concentration was increased during observation period ($P < 0.01$) and was greater in tie-stall housed cattle in comparison with loose housed animals (*P*

< 0.05) (Table 2). No factor or interaction influenced the fibrinogen concentration and the fibrinogen/total protein ratio (Table 2). The albumin concentration increased significantly in time ($P < 0.001$) (Table 2). The A/G ratio was influenced only by the time ($P < 0.01$): the ratio decreased on d 30 and d 60, while returned to the initial value on d 90 (Table 2). There was an effect of time ($P < 0.001$) and housing model ($P < 0.01$) for lysozyme. The lysozyme levels statistically decreased from d 0 to d 60 and returned to initial values on d 90 (Table 2). Moreover, it was statistically greater in cattle housed in tie-stall conditions in comparison with cattle housed in loose conditions (Table 2). The back transformation of the lysozyme least squares means (± 95% Confidence Interval) has been reported in Table 3. The SOD activity has been influenced by time ($P < 0.001$) and time x housing interaction ($P < 0.01$) (Fig. 1B). In particular, there was a statistically significant decrease on d 60 in comparison with d 0 and d 30. SOD values increased again on d 90, but remained statistically lesser than d 0 (Table 2).

3.3. Hormonal variables

Both time ($P < 0.001$) and housing model ($P < 0.05$) affected the cortisol concentration. There was a significant increase on d 60 and d 90 in comparison with d 0, while the cortisol level was higher in tie-stall housed cattle compared to loose housed animals (Table 2). There was only an effect of housing model ($P < 0.05$) for serum corticosterone concentration which increased in tie-stall housed cattle compared with loose housed animals (Table 2). Time ($P < 0.001$), housing model ($P < 0.05$) and time x housing interaction ($P < 0.05$) induced a significant change of faecal corticosterone concentration (Table 2; Fig. 1C). Particularly, the faecal corticosterone concentration increased significantly in time. Moreover, it was higher in cattle housed in tie-stall conditions compared with loose housed cattle (Table 2).

3.4. Quantitative expression analysis of GR-α by qPCR

Housing model ($P < 0.05$) influenced the GR-α gene expression which is greater in loose housed group compared to tie-stall housed group (Table 2). The back transformation of the GR-α gene expression least squares means (± 95% Confidence Interval) has been reported in Table 3.

4. Discussion

Many variables could be used to provide information about the stress conditions of cattle. Changes in the populations of WBC types (lymphocytes and neutrophils) in response to stressors, particularly the relative decrease in lymphocytes compared with neutrophil number, have been measured in studies relevant to immune function in health and susceptibility to infections in the bovine (Burton et al., 2005). Alterations in circulating leukocyte subsets are common findings in stressed animals and they have been documented in beef cattle under various management practices such as restricted space allowance during housing of steers, transportation of bulls (Buckham Sporer et al., 2008) and weaning of calves (Lynch et al., 2010b). In this study the lymphocytes course and N/L ratio were not affected by time or housing. This data could be explained by sampling time, since the monitoring of beef cattle began one months after the composition of the experimental groups. In this period of time these parameters, even if modified, could have been returned to baseline as previously reported (Lynch et al., 2010a; Earley et al., 2012).

Although the neutrophils are crucial for the control and elimination of many pathogens during an inflammatory response, they can also damage host tissues by rapidly degranulating and releasing proteolytic enzymes and reactive oxygen species. Markers of oxidative status are important because of their role in the pathways that link oxidation to pathologic processes. SOD is considered to be the

first defence against pro-oxidants. In our study the decrease of neutrophil number was not significant, while the SOD activity decreased and remained statistically lower than the initial value. According to Pigeolet and colleagues (1990), the reduction in SOD activity observed in the present study may arise from the susceptibility of SOD to oxidative reactive molecules. No difference between loose and tie-stall housed cattle has been detected. The antioxidant status is the result of the interplay of enzymatic and non-enzymatic elements with the systemic metabolic interactions (Ghiselli et al., 2000). Moreover, in ruminants the activity of antioxidant compounds can be influenced by nutrition and seasons (Bernabucci et al., 2002; Gatellier et al., 2004; Descalzo et al., 2005). Therefore, the measure of a single variable as performed in our study could not be sufficient to point out the degree of the oxidative stress.

Further information about the functional activity of neutrophils can be obtained from the measurement of serum lysozyme. A lack of lysozyme is correlated with a substantial decreased ability of the immune system to cope with environmental pathogens. Although the observed blood concentrations of lysozyme in this study may be considered physiological (Amadori et al., 1997), they statistically changed in time and were affected by housing conditions.

Stressful conditions are known to stimulate the secretion of catecholamines and GCs from the adrenal medulla and adrenal cortex, respectively (Ehrhart-Bornstein and Bornstein, 2008). Acute elevation of plasma of GCs, in particular cortisol, is primarily an adaptive mechanism to cope with stress by mobilising body reserves. For this reason, among GCs circulating cortisol is currently the most predominant measure of stress in cattle. The GCs increase together with the suppression of immune function through the suppression of lymphocyte blastogenesis (Murata, 1997) and interferon gamma (IFN-γ) production (Cheville, 2006) are key defining features of the stress

response in cattle. The effect of rearing conditions on serum cortisol concentrations has been investigated in bovine (Gupta et al., 2007); solitary housing conditions are known psychological stressors to cows (Rushen et al., 1999), and restricted space allowances increase acute plasma cortisol concentrations in bulls (Gupta et al., 2007). In our study, serum cortisol and corticosterone levels were significantly higher in tie-stall housed animals. This result has been sustained by concomitant significant lesser expression of GR-α in tie-stall housed animals. This inverse relationship presumably provides a short-loop feedback mechanism to protect cells against prolonged exposure to GCs. However, the glucocorticoid receptor down-regulation in lymphocytes of stressed animals has been reported by many authors (Preisler et al., 2000a; Preisler et al., 2000b; Burton et al., 2005).

In the past years, the marked variability in serum cortisol concentrations due to circadian rhythms and handling led to the development of methods for detecting GC metabolites excreted in faecal samples. Several studies have established that the measurement of faecal GC metabolites is a reliable and useful indicator of acute adrenal activity in cattle (Palme et al., 2000). Corticosterone is a major indicator of stress and is the major stress steroid produced in non-human mammals. Measuring faecal cortisol metabolites as an indicator of adrenocortical activity in animals offers the advantage of a simple sampling technique that will not interfere with the results of the study. Our data showed that faecal corticosterone concentration significantly increased in tie-stall animals, confirming the results obtained by serum analysis.

The levels of circulating cortisol detected in this study could also explain the increase in time of total protein and albumin concentration, as previously reported in stressed ruminants (Parker et al., 2003). Indeed, the total protein concentration as cortisol levels was greater in tie-stall housed animals.

Therefore, the differences observed in total protein, lysozyme, cortisol, serum and faecal corticosterone concentration and GR-α gene expression suggest that tie-stall housed cattle were more stressed than loose housed animals.

5. Conclusion

The stress caused by tie-stall housing has already been demonstrated in horses (Hoffmann et al., 2012) and in dairy cows (Popescu et al., 2013). In this study several parameters have been evaluated as possible indicators of stress in beef cattle. In conclusion, our results indicate that the housing system (loose housing *vs* tie-stall) can affect the haematological and physiological parameters in Piemontese beef cattle. All these alterations, when considered together, may be effective biomarkers of stress and therefore of disease susceptibility. Early detection of stressed animals may be of help to prevent disease severity and incidence, with proper action.

Conflict of interest

The authors declare no conflict of interest.

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Table 1

Primers sequences used in real-time PCR.

Gene	Sense	Sequence 5’ → 3’	Amplicon (bp)	GeneBank no.
GR-α	Fw	CCATTTCTGTTCACGGTGTG	132	AY238475
	Rev	CTGAACCGACAGGAATTGGT		
PPIA	Fw	GCCCCAACACAAATGGTT	95	NM_178320
	Rev	CCCTCTTTCACCTTGCCAAAG		

GR-α: glucocorticoid receptor α; PPIA = cyclophilin A.

Fw: forward; Rev: reverse.

bp: base pair

Table 2

The effect of time and housing system on haematological, biochemical, hormonal variables and GR-α fold gene expression of Piemontese beef cattle. The sampling was performed monthly for four times (0, 30, 60 and 90 days from the beginning of trial). The data are expressed as least squares means.

	Time (T)				Housing system (H)			*P*-values		
	d 0	d 30	d 60	d 90	loose	tie-stall	RMSE	T	H	TxH
WBC (x 10^3/µL)	8.44[a]	8.30[a]	8.17[a]	9.57[b]	9.11	8.12	0.24	*	NS	*
Neutrophil (x 10^3/µL)	2.99	2.58	2.99	3.38	2.91	3.05	0.18	NS	NS	NS
Lymphocyte (x 10^3/µL)	4.43	4.57	4.24	4.41	4.81	4.02	0.17	NS	NS	NS
N/L ratio	0.70	0.58	0.74	0.82	0.65	0.77	0.03	NS	NS	NS
TP (g/dL)	6.58[a]	6.88[b]	6.93[b]	7.18[c]	6.70	7.08	0.07	**	*	NS
Fibrinogen	583	586	612	593	576	610	28	NS	NS	NS

(mg/dL)										
Fibrinogen/ TP ratio	1.17	1.22	1.17	1.29	1.20	1.23	0.06	NS	NS	NS
Albumin (g/dL)	3.32[a]	3.67[b]	3.81[c]	4.00[d]	3.62	3.78	0.07	***	NS	NS
A/G ratio	0.84[a]	0.80[bc]	0.76[b]	0.81[ac]	0.82	0.78	0.01	**	NS	NS
Lysozyme (µg/mL)[§]	1.53[a]	0.40[b]	0.95[c]	1.69[a]	1.14	1.58	0.23	***	**	NS
SOD (U/mL)	30.39[a]	25.92[ac]	7.25[b]	21.38[c]	21.65	20.82	0.71	***	NS	**
Cortisol (ng/mL)	25.85[a]	27.67[ab]	28.18[b]	28.87[b]	21.64	33.66	1.83	***	*	NS
Corticosterone (ng/dL)	20.38	19.98	21.00	19.98	16.37	24.30	1.07	NS	*	NS
Faecal corticosterone (ng/g)	34.49[a]	36.92[ab]	38.84[bc]	44.12[d]	32.55	44.69	3.45	***	*	*

GR-α Fold increase ($2^{-\Delta\Delta Ct}$)[§]	-1.01	-1.85	-1.37	-1.06	-1.02	-1.62	0.24	NS	*	NS

T: time; H: housing system.

d: days from the beginning of trial.

RMSE: root-mean-square error.

WBC: white blood cells; N/L: neutrophil/lymphocyte; TP: total protein; A/G: albumin/globulin; SOD: superoxide dismutase; GR-α: glucocorticoid receptor α.

[§]Data expressed in logarithmic form.

Least squares means with different superscript letter (‘a’, ‘b’, ‘c’, ‘d’) differ for $P < 0.05$.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS: not significant.

Table 3

The back transformation of least squares means (± 95% Confidence Interval) of the lysozyme concentration and GR-α gene expression of Piemontese beef cattle. The sampling was performed monthly for four time times (0, 30, 60 and 90 days from the beginning of trial).

	Time (T)				Housing system (H)	
	d 0	d 30	d 60	d 90	loose	tie-stall
Lysozyme (µg/mL)	4.61 (3.66–5.80)	1.49 (1.04–2.13)	2.57 (2.15–3.08)	5.40 (3.89–7.50)	2.61 (2.09–3.25)	3.74 (3.00–4.66)
GR-α Fold increase ($2^{-\Delta\Delta Ct}$)	0.36 (0.29–0.45)	0.15 (0.07–0.34)	0.25 (0.15–0.42)	0.35 (0.26–0.47)	0.36 (0.26–0.50)	0.20 (0.14–0.27)

T: time; H: housing system.

d: days from the beginning of trial.

GR-α: glucocorticoid receptor α.

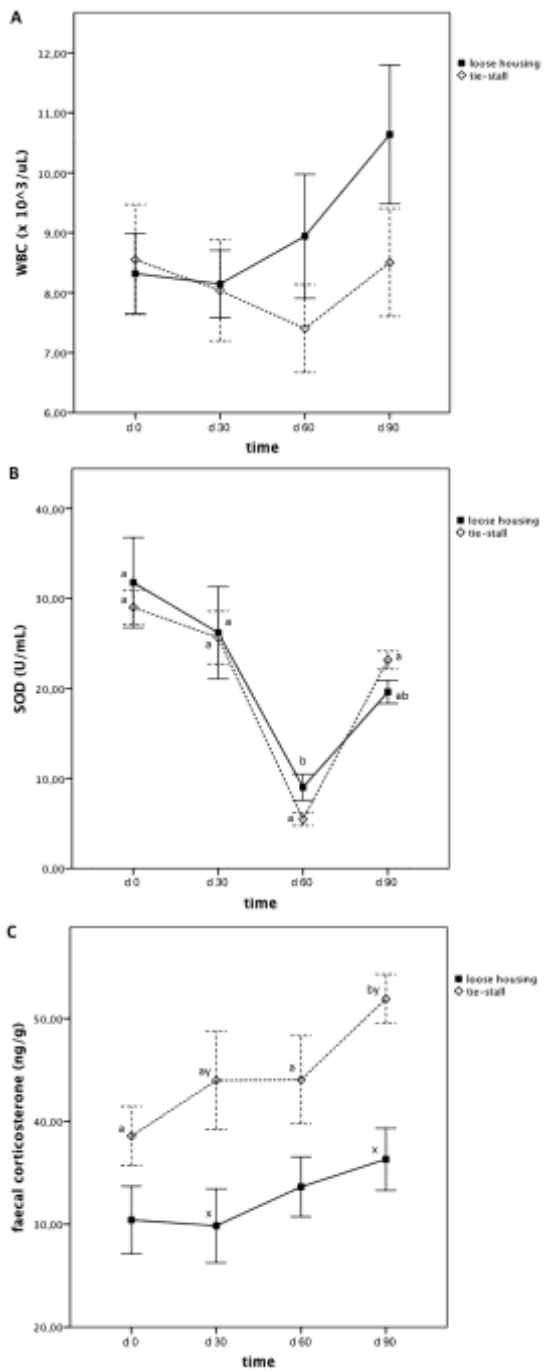

Figure 1

Fig. 1. WBC number (A), SOD activity (B), and faecal corticosterone concentration (C) in loose and tie-stall housed Piemontese beef cattle. The sampling was performed monthly for four times (0, 30, 60 and 90 days from the beginning of trial). The values are expressed as least squares means ± SE. [a,b] Least squares means with different superscript letter ('a', 'b', 'c', 'd') differ for $P < 0.05$. [x,y] Within each sampling time point, means without a common superscript letter differ $P < 0.05$. Time x Housing interaction $P < 0.05$. d: days from the beginning of trial.