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Plasma cortisol level in relation to welfare conditions in dairy farms

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SUMMARY – The cortisol is an index proposed to evaluate the stress status in dairy cows, but its interpretation remains puzzling. In particular, the actual basal levels remain difficult to establish, as any common cow/man interactions could raise it. To improve the knowledge about this potential index, cortisol level and some other blood parameters, were monitored in 10 commercial herds, characterized by 'good' and 'less good' welfare status. Using any care to avoid fright during cow capture, 8-10 lactating multiparous cows for each herd were bled. The bleeding was repeated 15 and 30 minutes later, leaving animals restrained in the rack. For statistical evaluation, cows of each herd were grouped according to their basal value of cortisol (<3, 3-10, >10 ng/ml) and cortisol increase (<3, 3-10, >10 ng/ml) after 1st bleeding. In addition, a retrospective grouping of herds, according to the average frequency of cows with less than 3 ng/ml at 1st bleeding, was realized: low (LPC), average (ALP) and high (HPC) plasma cortisol.
At 1st bleeding (basal), the cortisol showed the lowest values. Interestingly, 'good' herds exhibited higher frequency of cows with cortisol under 3 ng/ml (44 vs 29% of 'less good'). During the following 30' the cortisol raised, usually peaking at 15'. The herds separation according to basal cortisol level showed an obvious and significant (P<0.001) segregation between LPC, APC and HPC; moreover the responsiveness at bleeding challenge was higher in APC and HPC. Among performance and housing parameters checked, only age and, to some extent, stage of lactation, BCS, feed bunk space and free stalls availability seem related to cortisol level and/or its raise.
Basal plasma cortisol, when correctly measured, but also its post stress changes, seem related to the aggregate factors of discomfort of cows; nevertheless a more objective evaluation of welfare is needed to clarify the relationship between cortisol and chronic stress.

Key words: plasma cortisol, welfare evaluation, chronic stress, dairy cows.

INTRODUCTION – The increase of blood cortisol is a common effect of the acute stress (Sapolsky, 1992), and represents the final consequence of hypothalamic pituitary-adrenocortical axis activity. The role of this hormone is generally positive, at least immediately, improving fitness by energy mobilization (Raynaert et al., 1976), but it is not completely understood (Sapolsky et al. 2000).
A measurement of plasma cortisol is frequently used to study stress response (Sapolsky et al., 2000). Nevertheless its utilization as a stress indicator requires some caution because its level change according to several factors: type and severity of stressors (Mendoza et al., 2000; Bertoni et al., 2005a), circadian rhythms (Möstl and Palme, 2002), blood sampling (Negrão et al., 2004) as well as non-aversive events (Manteca, 1998). Therefore, the interpretation of cortisol levels remains a puzzling.
In particular, according to Mendoza et al. (2000), the higher cortisol level occurs after acute stress and it is maintained in case of failure to restore homeostasis following the acute response to stress or in case of repeated activation of the acute response, but in case of chronic stress the blood cortisol is not always high. Moreover, our understanding of long-term stress in animal is poor (Ladewig, 2000) and results in literature are contradictory. Von Borell (2001) stated that animals might adapt to long-acting housing conditions that are presumed to be stressful, suggesting a progressive reduction of blood cortisol during adaptation and, consequently, a higher blood level of this hormone till adaptation occurs. This agrees with the results of Smith and Dobson (2002), that observed a reduction of cortisol response after a prolonged stress input due to the habituation. For these reasons, it is widely recognized the assumption that chronic stress results in a hyper-reactivity of the adrenal cortex that justifies the use of blood cortisol level as indicator of physical and psychological stress in animals. Nevertheless, as reviewed by Manteca (1998) and at least for humans by Mendoza et al. (2000), the evidence supporting

these assumptions is far from to be conclusive.
As we have recently demonstrated on dairy cows (Bertoni et al., 2005a), an explanation to these contradictory results could be also related to the management and sampling techniques, that could induce a cortisol raise in blood. Nevertheless, the same research showed the possibility to evaluate the actual basal blood level of cortisol in dairy cows (also in field conditions), but only following well standardized conditions.
Aim of the present research was to improve the knowledge on the basal cortisol level as potential index of the chronic stress in field conditions and on the possible relationship with the spontaneous cortisol release associated to the stress stimulus of 1st bleeding and restraint.

MATERIAL AND METHODS – The trials were performed on lactating multiparous cows, belonging to 10 dairy farms of Po Valley with good genetic merit (on average over 90 ton/cow/lactation). The herds were chosen according to their welfare status, defined 'good' or 'less good' by the farm veterinary surgeon (vet) – the same for the 10 herds - according to the general cow behaviour. In particular the welfare was considered good when cows were quiet and easy to capture, while it was considered less good when cows were often restlessness and the capture was difficult. All the cows were housed in free barns, 8 with free stalls and 2 with bedded pack; all them were fed once a day by Total Mixed Ration (TMR).
About 3 hours after TMR distribution and far from any other operation, 8-10 cows for each herd were captured, using any care to avoid fright, and immediately bled from the jugular vein in heparinized tubes. Samples were immediately refrigerated in ice cold water until centrifugation. Bleeding was repeated exactly 15 and 30 minutes later, leaving cows restrained in the rack. The bleedings at every fixed time were carried out in a short lapse (not more than 5 minutes between the 1st and the last bled cow). A small aliquot of each blood sample was used to determine packed cell volume (PCV), while the remaining portion was centrifuged (3500 g per 16 min. at 5°C) and the plasma was divided in aliquots, stored at –20 °C until required for analysis.
Plasma cortisol was measured by RIA method using a commercial kit (Coat-A-Count DPC kit, Los Angeles, CA, USA). The coefficients of variation within and between assays ranged from 3 to 10%. The minimum detectable concentration was 1.4 ng/ml.
Plasma glucose, ceruloplasmin, haptoglobin, non esterified fatty acids (NEFA), potassium and paraoxonase were measured according to the methods described by Bertoni et al. (2005c), using an auto-analyser ILAB 600 (IL, MA, USA).
In addition to blood sampling, body condition score (ADAS, 1986), cleanliness score (Faye and Barnouin, 1985) and presence of injuries or diseases were evaluated on each cows, while parity, days in milk (DIM) and milk yield were also recorded. Finally, in each herd it was monitored the size of group, the availability of bunk space and of free stalls.
For statistical evaluation, cows of each herd were grouped according to their basal value of cortisol (<3, 3-10, >10 ng/ml) and for cortisol increase (<3, 3-10, >10 ng/ml) after 1st bleeding; then the herds were retrospectively divided in 3 similar groups according to their average frequency of cows with less than 3 ng/ml of cortisol at 1st bleeding, named low (LPC), average (APC) and high (HPC) respectively. Statistical analysis was performed by a two-way ANOVA (PROC GLM of SAS, version 8, TS M0), the model included herd, nested within group (LPC, APC, HPC), and time from 1st bleeding. Plasma cortisol was processed after logarithmic transformation. Moreover blood cortisol variations during restraint were evaluated by covariance analysis and as differences between 2 following bleedings (PROC GLM of SAS).

RESULTS – The main characteristics of 10 herds, as well as the judgment on welfare status of farm vet, are shown in table 1. Between the general parameters considered to classify the herds, only two are related to the vet judgment (good or less good); they are the group size, higher in 'good' group although the standard deviation is resulted quite large, and the cleanliness score, lower in 'good' group ($P<0.01$), that means cleaner cows.
Table 2 shows the average plasma cortisol immediately after the capture and their variations during the

30' of restraint in each herd as well as the frequency classes of cows with different basal cortisol level (<3, 3-10, >10 ng/ml) and the frequency classes of cows with low or high cortisol increase after 1st bleeding (<3, 3-10, >10 ng/ml at 15'). Plasma cortisol have shown the lowest levels at the 1st bleeding in all the herds (P<0.01 *vs.* 15 and 30 minutes), but the variability within herds was very marked also at the 1st bleeding. Fifteen minutes after the 1st blood sampling, the cortisol level had reached the peak, also with a large variability between herds, particularly in the "good" group. These cortisol values, as well as the frequency of cows within the herds characterized by low or relatively high values of blood cortisol – basal or response to stress values –, are only slightly different in the 2 groups suggested by the vet: average cortisol higher in "less good" group.

For this reason, the herds were retrospectively classified in 3 new groups, based on the average frequency of cows with less than 3 ng/ml of cortisol at 1st bleeding: low (LPC), average (APC) and high (HPC) (table 3).

Obviously, this subdivision have allowed a clear segregation between the herds, in fact the differences of plasma cortisol between all the groups have reached the statistical significance, particularly between LPC and HPC (P<0.001), that is confirmed by different percentage of cows with low cortisol basal values (<3 ng/ml) 64%, 36% and 10% respectively in LPC, APC and HPC groups. These marked differences are also confirmed in the next 2 bleedings, but only relatively to the LPC group in comparison to the others (P<0.001). Considering the differences between 2 following bleedings to exclude the basal value effect, APC showed the highest raise (P<0.01 vs LPC and P<0.05 vs HPC between 15' and 30').

Therefore, although the cortisol raise does not appear exclusively related to the basal value, LPC has shown the smallest increase after 1st bleeding. This result is also confirmed by the lowest percentage of cows with a marked cortisol increase (>10 ng/ml), only 5% in comparison to 43% of APC and 29% of HPC (table 3).

In table 4 some performance and housing parameters controlled in the herds are shown, grouped according to the average frequency of cows with less than 3 ng/ml of cortisol at 1st bleeding. These parameters were monitored to give an evaluation of the welfare conditions and therefore it is an attempt to find some relationships with the cortisol behavior. The major differences for the performance parameters are those regarding age, significantly higher in LPC respect to HPC (P<0.05), DIM and BCS with lower values in LPC; milk yield is otherwise very close. With concern to the housing parameters there are no statistical differences, but it clearly appears that HPC group is characterized by the worst conditions: lower availability of feed bunk space and free stalls (mainly due to herd 1), a tendency to have a bigger groups and more dirty animals.

Finally, among the checked plasma parameters the only observed differences are limited to glucose, higher in HPC (3.93 vs 3.79 mmol/l of LPC), haptoglobin, ceruloplasmin and paraoxonase also higher, but only as a tendency, in HPC herds.

DISCUSSION – The first interesting aspect of our results is the quite clear differentiation of the herds according to their basal plasma cortisol level. There are in fact herds with the largest part of the animals showing cortisol lower than 8-10 ng/ml and *viceversa* other herds where cortisol is often higher than 8-10 ng/ml. The cortisol values are not perfectly corresponding to the welfare judgment of the vet, but are in a better agreement with a more objective attempt to retrospectively define the welfare (tab. 4). Therefore, these results could agree with the general knowledge that chronically stressed animals have often a higher blood cortisol level (Mendoza et al., 2000). The study of cortisol level and its relationship with chronic stress require however two main points: the proper sampling of the cows to obtain the "true" basal level of cortisol and also the objective welfare evaluation. The first target is maybe obtained after our preliminary trials (Bertoni et al., 2005a); the second must be pursued more carefully to avoid what Lay and Wilson (2001) stated: "in some experiments the assumption of a chronic stress is attributed to a situation that is pre-conceived to be poor". For a better appraisal, in the future a new model for welfare evaluation of dairy herds, still in progress at our Institute (Calamari et al., 2004) will be utilized.

The second interesting aspect is that the responsiveness of the cows to the restraint-bleeding stress appears in some extent related to the basal level of cortisol; in fact the LPC cows have shown a rise of cortisol above 10 ng/ml in the 5% of cows vs. 43 and 29% of APC and HPC. These results disagree with an other our research (Bertoni et al., 2005a), but are in agreement with that of Weiss et al. (2004), both obtained with the ACTH challenge. The results also agree with those of Broom (1988), indicating that chronically stressed animals have a higher reactivity of adrenal cortex.

CONCLUSIONS – Cortisol values in blood can be affected by many factors (daytime, meal, worker operations, bleeding operations etc.), therefore it is worthy to pay attention to all of them if the true basal value of cortisol is the target. These basal values seem to be related to the welfare status, namely higher when herd conditions are "less good"; nevertheless a better and objective evaluation of the animal welfare seems essential for a more accurate study of such relationship.
A proper evaluation of the basal value seems also worthy if the responsiveness of the animal to the stress is needed; in fact, if the rise of cortisol does not reach the maximum level (Bertoni et al., 2005b), it is important to know the correct difference between post stress values and true basal values. Of course our data cannot guarantee that this procedure could be an acceptable one to measure the cow responsiveness, because the stimulation after bleeding-restraint stress is not so standardized as it could be with the ACTH challenge; nevertheless, the promising relationship between true basal level and the following increase seems to encourage the use of this approach on welfare evaluation.

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Table 1 – Main characteristics of herds grouped for 'good' and 'less good' welfare according to vet judgment.

Herd	*welfare vet judgment*	*cheked cows (n°)*	*parity (n°)*	*DIM*	*BCS*	*Milk yield (kg/d)*	*CS*	*group size (n°)*	*feed bunk space/cows*	*free stalls/cows*
1	**good**	7	2.6	65	2.00	37.8	1.00	154	0.7	0.8
4		8	2.1	273	2.29	22.4	0.90	150	1.0	1.0
6		8	3.4	119	2.20	37.4	0.50	140	0.9	1.0
9		7	2.7	179	1.96	28.0	0.65	24	1.0	1.0
10		8	3.3	131	2.00	35.9	1.00	55	1.0	1.0
	average	**7.6**	**2.8**	**153.4**	**2.1**	**32.3**	**0.8**	**104.6**	**0.9**	
	ds	**0.5**	**0.5**	**77.9**	**0.1**	**6.8**	**0.2**	**60.6**	**0.2**	
2	**less good**	6	2.2	386	2.47	28.8	1.25	61	1.1	6.4 (*)
3		7	2.3	96	2.25	30.2	1.50	70	0.9	1.0
5		7	2.9	174	2.13	35.4	1.20	60	1.5	1.0
7		8	3.4	125	1.95	28.2	1.65	58	0.9	4.5 (*)
8		7	3.6	95	2.19	36.3	1.20	96	0.9	0.9
	average	**7.0**	**2.9**	**175.1**	**2.2**	**31.8**	**1.4**	**69.0**	**1.0**	
	ds	**0.7**	**0.6**	**122.1**	**0.2**	**3.8**	**0.2**	**15.8**	**0.3**	

Note: (*) bedded pack (available m^2/cow); CS = cleanliness score

Table 2 – Average pattern of change of plasma cortisol during 30' of restraint and frequency of cows grouped according to cortisol level (basal or 15' later). The herds were divided in 'good' and 'less good' welfare conditions according to vet judgment.

		Plasma cortisol (ng/ml)			**Frequency (%) of cows with different level of cortisol at basal time**			**Frequency (%) of cows with different increase of cortisol after 15'**		
		minutes			***ng/ml***			***ng/ml***		
herd	welfare vet assessment	***0***	***15***	***30***	***<3***	***3-10***	***>10***	***<3***	***3-10***	***>10***
1	**good**	6.96	15.32	15.32	0.0	71.4	28.6	14.3	57.1	28.6
4		6.64	21.98	22.94	37.5	37.5	25.0	37.5	0.0	62.5
6		4.22	9.69	6.65	50.0	37.5	12.5	50.0	37.5	12.5
9		2.47	4.37	3.40	71.4	28.6	0.0	71.4	28.6	0.0
10		2.97	7.83	6.33	62.5	37.5	0.0	25.0	75.0	0.0
	average	**4.7**	**11.8**	**10.9**	**44.3**	**42.5**	**13.2**	**39.6**	**39.6**	**20.7**
	ds	**2.1**	**6.9**	**8.1**	**27.9**	**16.6**	**13.5**	**22.2**	**28.5**	**26.1**
2	**less good**	11.43	14.36	11.89	16.7	16.7	66.7	66.7	33.3	0.0
3		7.06	18.43	10.39	14.3	57.1	28.6	0.0	42.9	57.1
5		4.56	19.81	19.15	42.9	42.9	14.3	0.0	28.6	71.4
7		6.06	13.21	8.90	12.5	75.0	12.5	12.5	62.5	25.0
8		4.35	11.54	7.54	57.1	28.6	14.3	28.6	57.1	14.3
	average	**6.7**	**15.5**	**11.6**	**28.7**	**44.1**	**27.3**	**21.6**	**44.9**	**33.6**
	ds	**2.9**	**3.5**	**4.5**	**20.2**	**23.0**	**23.0**	**27.8**	**14.7**	**29.8**

Table 3 – Average pattern of change of plasma cortisol (PC) during 30' of restraint and frequency of cows with different behavior of cortisol (basal or 15' later) in herds grouped according to low (LPC), average (APC) and high (HPC) frequency of cows with plasma cortisol under 3 ng/ml at the basal level (1st bleeding).

		Plasma cortisol (ng/ml)			Frequency (%) of cows with different level of cortisol at basal time			Frequency (%) of cows with different increase of cortisol after 15'		
		minutes			*ng/ml*			*ng/ml*		
		0	*15*	*30*	*<3*	*3-10*	*>10*	*< 3*	*3-10*	*> 10*
groups										
LPC	average	3.26	7.91	5.76	63.7	31.5	4.8	41.7	53.6	4.8
(3 herds)	ds	0.97	3.59	2.13	7.2	5.2	8.2	25.8	23.4	8.2
APC	average	5.37	16.17	14.41	35.7	48.2	16.1	25.0	32.1	42.9
(4 herds)	ds	1.16	5.71	7.87	16.3	18.0	6.0	22.8	25.8	28.5
HPC	average	8.48	16.03	12.53	10.3	48.4	41.3	27.0	44.4	28.6
(3 herds)	ds	2.55	2.13	2.53	9.0	28.4	22.0	35.1	12.0	28.6

Table 4 – Some performance and housing parameters controlled in herds grouped according to low (LPC), average (APC) and high (HPC) frequency of cows with plasma cortisol under 3 ng/ml at the basal level (1st bleeding).

Herd	*welfare vet judgment*	*cheked cows (n°)*	*parity (n°)*	*DIM*	*BCS*	*Milk yield (kg/d)*	*CS*	*group size (n°)*	*feed bunk space/cows*	*free stalls/cows*
LPC	poor	7	3.6	95	2.19	36.3	1.20	96	0.9	0.9
	good	7	2.7	179	1.96	28.0	0.65	24	1.0	1.0
	good	8	3.3	131	2.00	35.9	1.00	55	1.0	1.0
	average	**7.3**	**3.2**	**135.1**	**2.05**	**33.4**	**0.95**	**58.3**	**0.96**	**0.97**
	ds	**0.6**	**0.4**	**42.3**	**0.12**	**4.7**	**0.28**	**36.1**	**0.07**	**0.06**
APC	good	8	2.1	273	2.29	22.4	0.90	150	1.0	1.0
	poor	7	2.9	174	2.13	35.4	1.20	60	1.5	1.0
	good	8	3.4	119	2.20	37.4	0.50	140	0.9	1.0
	poor	8	3.4	125	1.95	28.2	1.65	58	0.9	4.5 (*)
	average	**7.8**	**2.9**	**172.5**	**2.14**	**30.8**	**1.06**	**102.0**	**1.06**	**1.00**
	ds	**0.5**	**0.6**	**71.1**	**0.15**	**6.9**	**0.49**	**49.8**	**0.30**	**0.00**
HPC	good	7	2.6	65	2.00	37.8	1.00	154	0.7	0.8
	poor	6	2.2	386	2.47	28.8	1.25	61	1.1	6.4 (*)
	poor	7	2.3	96	2.25	30.2	1.50	70	0.9	1.0
	average	**6.7**	**2.3**	**182.5**	**2.24**	**32.2**	**1.25**	**95.0**	**0.88**	**0.90**
	ds	**0.6**	**0.2**	**176.8**	**0.23**	**4.9**	**0.25**	**51.3**	**0.23**	**0.14**

Note: (*) bedded pack (available m^2/cow); DIM = day in milk CS = cleanliness score