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Some physiological and biochemical methods for acute and chronic stress evaluation in dairy cows

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ABSTRACT

Stress factors are so numerous and so diverse in their strength and duration that the consequences on animal welfare can be quite varied. The first important distinction concerns the characterization of acute and chronic stress conditions. Acute stress is a short-lived negative situation that allows a quick and quite complete recovery of the physiological balance (adaptation), while chronic stress is a long lasting condition from which the subject cannot fully recover (maladaptation). In the latter case, the direct effects of the stress factors (heat, low energy, anxiety, suffering etc.), as well as the indirect ones (changes occurring at endocrinological, immune system or function level) can be responsible for pre-pathological or pathological consequences which reduce animal welfare. To evaluate the possible chronic stress conditions in single animals or on a farm (in particular a farm of dairy cows), some parameters of the direct or indirect effects can be utilised. They are physiological (mainly hormone changes: cortisol, β-endorphin), behavioural (depression), biochemical (metabolites, acute phase proteins, glycated proteins etc.), as well as performance parameters (growing rate, milk yield, fertility, etc.). Special attention has been paid to the interpretation of cortisol levels and to its changes after an ACTH challenge. Despite fervent efforts, well established and accepted indices of chronic stress (distress) are currently lacking; but without this objective evaluation, the assessment of animal welfare and, therefore, the optimization of the livestock production, could prove more difficult.

Key words: Chronic stress, Physiological indices, Biochemical indices, Welfare evaluation.

RIASSUNTO

METODI BIOCHIMICI E FISIOLOGICI PER LA VALUTAZIONE DELLO STRESS ACUTO E CRONICO NEGLI ALLEVAMENTI BOVINI

I fattori di stress sono molto numerosi e differente è pure la loro potenza e durata, per tale ragione le conseguenze sulle condizioni di benessere animale possono essere completamente diverse. La prima importante distinzione a proposito di stress riguarda la caratterizzazione delle condizioni di stress acuto...
e cronico. Per stress acuto si intende una situazione negativa di breve periodo che consente un rapido e pressoché completo recupero dell’equilibrio fisiologico (adattamento). Per stress cronico invece, si intende quella condizione di lungo periodo che non permette un completo recupero da parte del soggetto (maladattamento). In questo secondo caso, le conseguenze dirette dei fattori di stress (ipertermia, carenza energetica, ansietà, sofferenza, ecc.), ma anche quelle indirette (cambiamenti dell’assetto endocrino e del sistema immunitario oppure del livello funzionale) possono essere responsabili di condizioni pre-patologiche o patologiche che determinano una diminuzione del livello di benessere. Per valutare la possibile situazione di stress cronico nei singoli soggetti o a livello di allevamento (in particolare di bovine da latte) possono essere utilizzati alcuni indicatori che misurano gli effetti diretti o indiretti di tale condizione. Tali indicatori sono di varia natura: fisiologici (principalmente variazioni ormonali: cortisolo, β-endorfinea), comportamentali (depressione), biochimici (metaboliti, proteine di fase acuta, proteine glicosilate, ecc.), ma anche parametri legati alle performance (incrementi ponderali, produzione di latte, fertilità ecc.). Una particolare attenzione è stata dedicata all’interpretazione dei livelli di cortisolo ed alla sua variazione a seguito della stimolazione del surrene con ACTH. Nonostante le molteplici ricerche effettuate, mancano ancora degli indicatori di stress cronico condivisi ed accettati dalla comunità scientifica; tuttavia senza di essi resta ardua sia la valutazione oggettiva del benessere animale che l’ottimizzazione delle produzioni zootecniche.

Parole chiave: Stress cronico, Indicatori fisiologici, Indicatori biochimici, Valutazione del benessere.

Introduction

After a prolonged and complex debate concerning animal welfare, following cultural developments of the societal view regarding the relationship between man and farm animals, the European scientific community has proposed an agreement on its definition (EFSA, 2007; Carenzi and Verga, 2009). However, the objective evaluation of animal welfare remains a complicated task for scientists despite various approaches that have been proposed. Sometimes a life without stress is considered essential to welfare, otherwise it is fundamental to clarify the relationship between a low level of welfare and stress, which are not always related phenomena. In particular, the explanation of what type of stress could negatively affect welfare conditions is quite basic.

With this aim, the first part of the review describes the circumstances - namely stress conditions - that determine a reduction in the welfare status, and, thereafter, more reliable indices to detect and to measure those conditions in farm animals are presented.

Stress

Eustress and distress

Stress is defined as a complex multidimensional phenomenon promoted by several noxious or unpredictable stimuli (stressors) that causes a physiological response (stress) aimed to maintain or to recover the body homeostasis (Moberg, 1980). Therefore, not all types of stress are harmful or even negative. In several cases stress is perceived as a neutral condition or as a positive condition (eustress) and maintains life in a pleasurable and stimulating manner. On the contrary, the stress causing negative effects is defined distress (Webster, 1983) and occurs when adaptation is not possible and causes a reduction in well-being (Broom, 2003). Whatever the animal perceives, the response begins with the activation of the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal system (HPA), but several other functions of the animal can be involved (e.g. immune system, metabolic pathways). However, to elicit a distress
response, a stressor must be perceived as dangerous factor by the animal, causing deleterious effects (Moberg, 2000). The perception can be vary depending on the genetic background, previous experiences, the psychological context of the stress (Mormède and Dantzer, 1998; McEwen, 1998; Romero, 2004; Kosti et al., 2006), and the age (Romero, 2004), the types and the duration of the stressors. Based on these the final response will also vary. In any case, a cascade of physiological adaptive (allostatic) responses are involved in the attempt to cope with the adverse stimuli occurring within the environment. As a final result, these adaptive modifications can maintain (eustress) or worsen (distress) the homeostasis.

**Acute and chronic stress**

Stress can be caused by different types of stressors that Sapolsky et al. (2000) have classified as: acute, sequential, episodic, chronically intermittent, sustained or anticipated. The stress could be well described in two main conditions in relationship to the duration of the consequent effects: transitory (acute) or long-term (chronic) stress. In any case, once the central nervous system perceives a threat, it develops a response that consists of some combination of the four general biological defence responses: behavioural, autonomic nervous system, neuroendocrine and immune (Moberg, 2000). Although all four biological defence systems are available for the animal to respond to a stressor, not all four are necessarily utilised against all the stressors. In particular, the homeostasis is maintained if only the first two defence mechanisms are involved; conversely, when all the defence mechanisms have been implicated some biological functions can be adversely modified and animals will be in distress.

Acute stress occurs consequent to a short-lived negative situation, either physical, emotional or psychological, that - generally - allows a quick and complete recovery of the physiological balance; thus the final result is a complete adaptation. Therefore, this condition is simple to define (but not to measure), because it causes a quick biological response in the animal. During an acute stress response (Figure 1), SNS and HPA axes are activated and many hormones are modified with a very short lag (a few seconds or minutes) and are increased (catecholamines, ACTH, opiates, vasopressin, prolactin, glucagon, GH and glucocorticoids) or reduced (e.g. serotonin, gonadotrophins). In addition, other physiological and metabolic effects can occur immediately or with some delay. Among these, the most common are an increased heart rate, a quickened breathing rate, higher blood pressure, an increased energy mobilization (rise of glucose and NEFA), the stimulation of the immune function and the reduction in appetite (Sapolsky et al., 2000). Other changes have also been observed, such as the reduction of some plasma minerals (potassium by Trevisi et al., 1992 and Halperin and Kamel, 1998; magnesium by Seeling, 1994), a slowed digesta flow rate in the rumen (Trevisi et al., 2007) and stomach (Taché et al., 1999), but an increase in the flow rate in the intestine (Taché et al., 1999; Mayer, 2000). Although the changes of several indices are very pronounced during acute stress conditions, most of them are detectable only in a short period following the events. When the stress ceases, the SNS and HPA systems are not further stimulated and the involved effectors or the hormones turn to baseline levels (recovery). In some cases, the adaptive homeostatic responses observed during acute stress can improve welfare. Therefore, the biological and physiological changes induced by acute stress cannot be used as...
markers for welfare evaluation. Nevertheless, as well explained by Moberg (2000), distress can also result from acute stress, if the biological cost is sufficient to alter biological functions (e.g. by disrupting critical biological events or by diverting resources away from other biological functions).

Unlike acute stress, chronic stress is a state of ongoing physiological arousal (Mendoza et al., 2000). This occurs when the body experiences so many stressors or repeated exposure to the same acute stress or continuous stress that the autonomic nervous system rarely has a chance to activate the relaxation response (e.g. the prolonged exposure to stress causes a permanent change in the level of one or more parameters in comparison to the usual ones). In this situation, there is overexposure to stress hormones that results in an “allostatic load” as postulated by McEwen (1998) and results in a biologic cost sufficient to alter biological functions and to induce distress (Moberg, 2000). In this condition the adaptation is not successful (maladaptation) and McEwen (1998) describes 4 situations causing it:

- frequent stress (recurrent activation and inactivation of stress systems);
- lack of adaptation to repeated stressors of the same type;
- the inability to shut off allostatic responses after a stress is terminated and
- inadequate responses by some allostatic systems trigger compensatory increases in others.

These different situations affect the physiological response and the last two potential modifications are illustrated in Figure 2. Chronic stress coincides with a long lasting state (e.g. life style or serious long illness, which has a constant and extreme effect on the emotional status and/or the metabolic status) from which the subject cannot fully recover and whose intensity and duration of suffering contribute to the severity of the final animal response (Webster, 1994). This long-term over stimulation of coping responses cause direct (heat, low energy, anxiety, suffering etc.) and indirect
effects (changes occurring at endocrine, immune, metabolic as well as specific function level). These effects are responsible for pre-pathological or pathological consequences which reduce welfare (Moberg, 2000; Romero, 2004). Therefore, chronic stress is a condition of maladaptation that can be associated with a direct reduction in the level of welfare. Furthermore, this condition can affect the onset or the susceptibility to disease and the progression of disease, even when there is another cause of the illness.

The measurement of chronic stress condition is more arguable than that of acute stress and, as suggested by Ladewig (2000), currently “we do not have specific tests or other methods that can be used to diagnose chronic stress”. In general, a good biological marker of chronic stress “must lead to subtle and long-term changes in a physiological function (e.g. endocrine, metabolic, immune systems) even if individuals have seemingly accepted the condition of their lives” (Kelly et al., 1997).

Markers of chronic stress

A variety of behavioural, physiological, biochemical, immunological and pathological parameters have been proposed as candidates of the animal responsiveness under chronic stress and, therefore, useful in objectively evaluating animal welfare (Table 1). Among these markers, several are not

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**Figure 2.** Normal allostatic (circle), prolonged (square) and inadequate (triangle) response to a stressor. In the normal allostatic the response is initiated by a stressor, sustained for an appropriate interval, and then turned off; in the prolonged the shutdown is delayed, while in the inadequate the lower response could be due to a compensatory hyperactivity of other mediators (adapted from McEwen, 1998).
measurable in a short time (i.e. indicators of the normal behaviour or incidence of pathologies) and, in addition, some could be measured only with qualitative methods (e.g. behavioural indicators). Physiological and biochemical indices seem to be more reliable and objective, although the knowledge concerning several of these parameters seems controversial or not exhaustive.

A possible explanation for the lack of agreement on the use of these indices is the unsatisfactory standardisation. Whatever the parameter, it is, in fact, necessary to ascertain its basal levels and how it fluctuates over the time (Broom, 2003) and according to the common conditions in an animal’s life (e.g. circadian cycle, stage of lactation, age, meal distribution, milking and other farm handling). For this reason, before suggesting the use of a certain welfare indicator, accurate studies to ascertain the basal values are needed. Obviously, the most useful indices are those that show the lowest variations with respect to the basal value in response to the common life conditions, but the highest changes in response to a chronic stressor. Kelly et al. (1997) proposed a list of the most important criteria for the ideal marker of chronic stress response in humans which include the following characteristics: (i) affected/caused/created basically by (di)stress; (ii) has a long half-life; (iii) does not require repeated measure; (iv) is independent of demographic factors (e.g. sex or age); (v) is easy and cheap to measure and (vi) does not require a special sample preparation. We can assume these criteria are also largely pertinent to animals, although few biological indicators have the previous requisites.

**Endocrine indicators**

As previously mentioned, during stressful situations two main endocrine systems are activated (Moberg, 2000); they promote the release of several hormones: catecholamines, namely epinephrine and norepinephrine, (SNS/adrenal medulla axis) and corticotrophin-releasing hormone=CRH → adrenocorticotrophin=ACTH → corticosteroids, namely cortisol (HPA axis). Unfortunately, most of these hormones show a very short spike in the blood after stress events, revealing their unreliability in measuring chronic stress conditions.

*Cortisol*

Cortisol - both basal level and/or its variation after ACTH challenge - is a possible candidate for chronic stress evaluation. Although an increase in cortisol occurs after any acute stress, it shows marked and more prolonged variations before and during distress that seems reasonably related to maladaptation (McEwen, 1998; Lay and Wilson, 2004). In fact high levels of cortisol seem to be maintained when there is a failure to restore homeostasis or after repeated stress (Figure 2). However, some contradictory results emerge from the literature relating to farm animals. Broom (1988) observed a hyper-reactivity in animals under chronic stress after ACTH challenges and supports the relationship between high basal cortisol levels and chronic stress, while Weiss et al. (2004) consider the hyper-reactivity under ACTH challenges to be true in pigs, but not in cattle. Undoubtedly, the interpretation of the basal blood cortisol is not easy because it is affected by a variety of factors, including the following: circadian rhythms (Möstl and Palme, 2002); sampling (Negrão et al., 2004); restraint (Bertoni et al., 2005a); stage of lactation (Bertoni et al., 2006a); coitus and nursing (Manteca, 1998); milking (Bertoni et al., 2005a; Rushen et al., 2007), degree of habituation (von Borell, 2001; Smith and Dobson, 2002), other hormones (e.g. vasopressin can potentiate ACTH secretion; Rushen et al., 2007).
In our experience, using a well standardised bleeding condition, the basal plasma cortisol of dairy cows in commercial herds seems linked to chronic stress and, consequently, to welfare conditions (Trevisi et al., 2005). The higher average values of cortisol have been observed in chronically stressed herds, selected with the model proposed by Calamari and Bertoni (2009) which is suggested to objectively evaluate the welfare situation of dairy farms. This relationship was confirmed in another trial where the basal cortisol level and its increase during restraint appear to be related (Trevisi et al., 2005). These data support the hypothesis that chronically stressed cows, when exposed to a mild stress, produce a higher reactivity of adrenal cortex, as suggested by Broom (1988).

To overcome the difficulties related to the basal blood cortisol interpretation (e.g. avoiding the variations due to blood sampling or restraint stress) some authors have proposed detecting it in different biological materials, such as saliva (Cooper et al., 1989), milk (Shutt and Fell, 1985; Verkerk et al., 1998), faeces (Morrow et al., 2002; Möstl and Palme, 2002) and urine (Hay and Mormede, 1998). The level of the cortisol is in some equilibrium with the different compartments and the sampling of these materials is, generally, considered less invasive. In these materials, detection and interpretation are not so simple for several reasons. First of all, the level could be lower than in blood (e.g. about 10 times less in saliva; Negrão et al., 2004); the hormone could be conjugated before excretion (e.g. urine and faeces, Möstl and Palme, 2002) and, again, transformed by bacteria in the gut; furthermore, the level could also show some fluctuations (e.g. faeces, Möstl and Palme, 2002) and the excretion be delayed (e.g. about 12 hours in the faeces), although this last point has no relevance during chronic stress because the expected levels are supposed to be permanently high. Therefore, the available data still remain unsatisfactory, despite some efforts toward standardisation (e.g. cortisol metabolites detection in the faeces, Möstl et al., 2002; Morrow et al., 2002). Among these different biological materials, the evaluation of cortisol (or its metabolites) in the faeces seems more promising. It provides an integrated measurement of the hormone production over an extended time period. For these reasons, Morrow et al. (2002) have proposed the use of this non invasive technique for monitoring health and welfare in dairy cattle, but in combination with other physiological and behavioural measures. However, these results are not yet very clear and further studies are necessary before replacing the cortisol determination in blood, which remains the most popular physiological index.

In addition to the basal level of cortisol, much attention has been given to the reactivity of the adrenal gland after an ACTH challenge, although the consequent level of plasma cortisol remains a controversial point. In this regard, it seems helpful to consider the results obtained with the recognized method of evaluation of the adrenal cortex sensitivity in vertebrates: the challenge with ACTH (Verkerk et al., 1994) or its analogue (ACTH 1-24 or tetracosactide). ACTH administration mimics a usual adrenal stress response by causing a quick rise in circulating cortisol, followed by a return to the baseline within few hours. Although this challenge is often used, it is applied with different protocols: dose, times of bleedings, response measurements (e.g. peak of cortisol, rate of increase, time to return to basal level). The data available in literature have been recently reviewed by Rushen et al. (2007) and confirm the complexity of the
HPA system. The sensitivity of the adrenal gland increases during the first days of repeated sequential stress (prolonged rise of cortisol level after ACTH challenge), while it tends to be reduced (quick reduction of cortisol level after ACTH challenge) thereafter. This means that – according to previous authors - repeated acute stress (daily ACTH challenge) does not necessarily evolve in chronic stress, when animals show an adaptation and therefore the plasma cortisol gradually decreases; contrariwise in the case of maladaptation the adrenal sensitivity remains sustained and, consequently, the cortisol level would be higher. The results are not conclusive and the changes in cortisol level after ACTH challenge could depend on the magnitude of the stress and on the interval between subsequent stressors. In this context and according to Rushen et al. (2007), some results from literature do not seem so contradictory. In particular, data from Ladewig and Smidt (1989) have shown a lower release of cortisol in response to ACTH injections in bulls apparently adapted to stress conditions (4 weeks after tethering). This suggests a reduced sensitivity of the adrenal cortex to ACTH challenge. On the contrary, Munksgaard and Simonsen (1996) did not observe a reduction in cortisol release in dairy cows after prolonged stress conditions (3 weeks of deprivation of lying down for 14 hours per day or social isolation). The first situation could simply represent a case of adaptation to stress, while the second one could be a case of chronic stress. However, not all the researchers agree with this idea and suggest that chronic stress “would result in an initial increase in sensitivity of the adrenal gland followed by a decrease” (Rushen et al., 2007).

These puzzling results regarding the cortisol level after ACTH challenge could be justified, analogously to their changes of the basal level, by several factors able to affect the adrenocortical response. The major factors include the following: environmental (season, temperature), physiological (milk yield, age, and stage of lactation; Hasegawa et al., 1997; Bertoni et al., 2006a) and genetic (Weiss et al., 2004; Kosti et al., 2006). One of more intriguing aspects has been shown by Kosti et al. (2006), who found an exaggerated ACTH release to restraint stress (30 minutes), but only a slightly higher (NS) rise of corticosterone, in a strain of rat characterised by more ‘anxious-like’ behaviour in comparison to a nonreactive strain. These results could in fact suggest a lower sensitivity of the adrenal gland during stress in ‘anxious-like’ subjects, but they could also imply the achievement of a plateau of the plasma cortisol level, therefore independent – above a certain threshold - to the degree of the ACTH stimulus. This last hypothesis has been partly demonstrated in our laboratory (Bertoni et al., 2005b) on dairy cows challenged with different amounts (20 to 1000 mcg) of synthetic ACTH (tetracosactrin, ACTH1-24). Surprisingly, the highest (1000 mcg) and the lowest (20 mcg) doses induce very similar increases in rates and maximum levels of plasma cortisol, if the peak is considered; otherwise higher doses are responsible for more prolonged high values.

The poor standardisation of the ACTH challenge (e.g. dose, times of bleeding and response measurement) seems, therefore, the most important cause of result variability. From our experience (Bertoni et al., 2005b), the following standardised conditions seem to be recommendable: (i) to inject very low dosage of ACTH (only 20 mcg/injection), because the maximum levels of cortisol – within each subject – do not seem to differ at higher dosages of ACTH; (ii) to check the blood cortisol until its re-
turn to the basal level (about 3 hours after the peak); (iii) to evaluate the area under the curve after the peak, which we suggest to be the real indices of the responsiveness of the animal to the challenge.

Using this procedure, our preliminary results show that cortisol response to ACTH challenge measured as the area under the curve:
• is higher in late lactation (P<0.001) and poorly affected by basal values (Bertoni et al., 2006a);
• is lower in fresh cows (30-40 days in milk), particularly if suffering from marked inflammatory events immediately after calving (Trevisi, unpublished data), which has been measured according to the Liver Functionality Index (Bertoni et al., 2006b). This lower level of cortisol in early lactating cows, could be a consequence of a lower transcortin (corticosteroid-binding globulin) synthesis in the liver, if challenged by a cause of acute phase reaction.

**β-endorphin**

The β-endorphin is a cleavage product of proopiomelanocortin, also a precursor of ACTH, formed in the anterior pituitary. It is supposed that β-endorphin produces a major effect in the CNS, where it interacts with the opiate receptors. For this reason, β-endorphin is involved in pain, cerebral ageing, behaviour with respect to feelings, food intake and emotions, which is why it is interesting as respects chronic stress evaluation. Although β-endorphin seems able to reduce perception of pain, similar to drugs such as morphine and codeine, Osawa et al. (2000) have reported an increase in this hormone in response to a variety of stressors. These authors found higher levels of β-endorphin at calving time in dairy cows with abnormal parturition (dystocia and or retained placenta) as compared to cows with normal puerperium. This suggests that more painful situations require a higher level of “narcotizing” hormones. Therefore, more data are needed to clarify the effective relationship between β-endorphin and chronic stress, for a better interpretation of its basal levels.

**Other hormone tests**

In addition to ACTH challenge, two other hormonal challenges have been proposed to evaluate chronic stress: changes in pituitary responsiveness to CRH stimulation (CRH test) and changes of CRH secretion due to glucocorticoids control (dexamethasone test). Some authors have applied these methods (Fisher et al., 2002), but the results do not seem encouraging for their utilisation in the evaluation of chronic stress.

**Physiological indicators**

Most of the affected physiological parameters (e.g. changes in the cardiovascular system, namely heart rate and heart rate variability; respiration rate; transpiration) have been mainly related to acute stress, suggesting limited interest as respects welfare evaluation (Table 1). Between these parameters, the measure of the heart rate variability (HRV) seems a promising approach for evaluating stress (acute and chronic) and for emotional states in human (Appelhans and Luecken, 2006) as well as in farm animals (von Borrel et al., 2007). HRV measures the continuous interplay between sympathetic and parasympathetic influences on heart rate, which determine the typical irregular time intervals between consecutive heart beats. Therefore, according to Appelhans and Luecken (2006), HRV represents the different capacity for regulated emotional responding.

In animals, HRV is proposed as an index of maladaptation for an expected future flight response, which could remain modified for several days during a psychologi-
Table 1. Main indices of acute and chronic stress in cattle. For the most acute stressors the biological cost is negligible because they are short-lived and, therefore, they do not affect the welfare condition.

| Indices | Reference |
|---|---|
| Behavioural indicators | vocalisation, restlessness, fight, pain, stop moving forward, "freeze" |
| | Broom (2003) |
| Physiological indicators | increase in heart rate, heart rate variability, blood pressure, respiration rate, transpiration (fear/arousal markers) |
| | Sapolsky et al. (2000); Broom (2003) |
| | increase in heart rate variability (fear/arousal marker) |
| | von Borrel et al. (2007); Mohr et al. (2002) |
| Performance indicators | reduction in milk yield |
| | Rushen et al. (2001) |
| interference with milk ejection | Bruckmaier and Blum (1998); Rushen et al. (2001) |
| | increase in incidences of diseases: infectious and metabolic |
| | Sheridan et al. (1994); Broom (2006); Broom and Fraser (2007) |
| Endocrine measurements | increase in CRH, catecholamines (epinephrine and norepinephrine), ACTH, cortisol, oxytocin, vasopressin |
| | Sapolsky et al. (2000); Broom and Fraser (2007) |
| | cortisol (basal level; changes after ACTH or CRH challenges) |
| | Munksgaard and Simonsen (1996); Weiss et al. (2004); Rushen et al. (2007); Bertoni et al. (2005b) |
| | increase in β-endorphin |
| | Sapolsky et al. (2000); Osawa et al. (2000); Broom (2006) |
| | increase in β-endorphin (?) |
| | Osawa et al. (2000) |

Continued >>
## Table 1. Continuation

| Immune indicators | Increase in neutrophil/lymphocyte ratio, monocytes, α-globulin; decrease of PBMC stimulations with mitogens | Broom (2003, 2006); Nemi et al. (1993) |
|-------------------|-------------------------------------------------------------------------------------------------|-------------------------------------|
|                   | Immunoglobulin M, hemolytic complement (alternative pathway)                                      | Lacetera et al. (2006)              |
|                   | Prolonged negative energy balance: increase in glycated proteins (e.g. hemoglobin, fructosamine) | Kelly et al. (1997); Tahara et al. (1995) |
| Biochemical markers | Increase in NEFA, BHB, urea and decrease in glucose (indices of food deprivation) | Sapolsky et al. (2000); Broom (2003) |
|                   | Increase in osmolality, PCV, total protein, albumin (indices of dehydration and/or haemoconcentration) | Broom (2003) |
|                   | Disease markers: increase in positive acute phase proteins (e.g. SSA, haptoglobin, ceruloplasmin); decrease of negative acute phase proteins (e.g. albumin, lipoprotein, RBP); decrease in PCV | Cappa et al. (1989); Colditz (2002); Arthington et al. (2003, 2005); Gruys et al. (2005); Murata et al. (2004); Bionaz et al. (2007); Piñeiro et al. (2007); Bortone et al. (2008); Lomberg et al. (2008) |
|                   | Increase in CK, lactate, LDH, LDH5/LDH, GOT (indices of physical exertion) | Broom (2003); Lay and Wilson (2004) |
|                   | Increase in PCV, glucose, urea, BHB (indices of fear/arousal and catecholamine release) | Broom (2003); Lay and Wilson (2004) |

**Abbreviations:** ACTH, adrenocorticotropin; ALP, Alkaline Phosphatase; BHB, Beta-HydroxyButyrate; CK, Creatinine Kinase; CRH, corticotropin-releasing hormone; GOT, Glutamic Oxaloacetic Transaminase; LDH, Lactate Dehydrogenase; LDH5, isoform of LDH; NEFA, Non Esterified Fatty Acids; PBMC, Peripheral Blood Mononuclear Cells; PCV, Packed-Cell Volume; RBP, Retinol Binding Protein; SSA, Serum Amyloid A.
cal stress (e.g. isolation), in comparison to cortisol and ACTH, which return to usual levels much more quickly. Von Borrell et al. (2007) have recently reviewed the potential usefulness of this non invasive technique in farm animals, mainly to evaluate chronic stress and welfare. These authors have emphasised that several parameters obtained from the statistical assay of HRV have been proposed as potential markers of chronic stress (Mohr et al., 2002); particularly in cattle, two of these parameters seem related to stress load (set of different stress conditions) and stress level (severity of the stress). The measurement of HRV remains, at present, too complex and expensive and requires further research.

Performance and health indices

The measures of performance (e.g. growing rate, body condition changes, milk yield, fertility) are not considered unanimously good indices of chronic stress; in fact some authors consider that higher performance (e.g. milk yield) is per se a stress condition or it increases the usual stress conditions. This appears questionable to us, suggesting that during chronic stress (and thus reduced welfare) the performance of cows could be in good agreement with their high genetic merit (Bertoni, 1999). On the contrary, physiological disturbances following chronic stress could determine marked effects on the metabolism that could be associated with a reduction in feed intake, to some changes of digestive channel physiology, to a negative energy balance, lipomobilisation and to an increase in the metabolic rate (Elsasser et al., 1995; Elsasser et al., 1997; Bertoni, 1999; Lay and Wilson, 2004).

All these effects will of course worsen the performance of dairy cows (Webster, 1983; Bertoni, 1999; Trevisi et al., 2001; Trevisi et al., 2003; Trevisi et al., 2006) as well as in beef cattle (SCAHAW, 2001; Broom, 2003). In our experience (Trevisi et al., 2001; Trevisi et al., 2003; Trevisi et al., 2006), in herds with chronic stress conditions (e.g. overcrowding, mistakes in the milking procedure and in the group handling, inadequate diet, etc.) mainly during the transition period, the dairy cows have produced less milk yield, of poor quality (less fat, less casein), and lose more body reserves. The performance indices (e.g. milk yield and quality, as well as BCS losses) cannot be evaluated according to absolute levels, but they have to be compared with the genetic merit of the reference population. For example, a certain milk yield level could be low for one breed, but too high for another one. Therefore, these parameters have to be assessed comparing their levels with those observed in an analogous reference population (e.g. same breed, similar genetic merit, but also with the same parity and the same stage of lactation).

Furthermore, under chronic stress, dairy cows are more susceptible to metabolic (Broom, 2006; Broom and Fraser, 2007) and infectious (Sheridan et al., 1994) diseases, and are often less fertile (e.g. increase in the days open and services per pregnancy, reduction in the number of pregnancies at the first insemination). Therefore, the health and fertility indicators can be linked with chronic stress for:

• the worsening of health status; three possible mechanisms have been proposed by Broom (Broom, 2006; Broom and Fraser, 2007): 1) the chronic activation of physiological coping mechanisms, that cause a immuno-suppression and, then, the occurrence of infectious diseases; 2) the behavioural coping mechanisms that cause injurious abnormal behaviour, often followed by physical injuries; 3) the possible metabolic stress, outcome of many factors (e.g. management, genetic selection, nutrition mistakes) that determines the
development of the metabolic “production diseases”;

- the reduction in female fertility; Sapolsky et al. (2000) and Dobson et al. (2001) have demonstrated that the exposure to chronic stress reduces the pulsatile patterns of GnRH (Gonadotrophin-Releasing Hormone) release and LH (luteinizing hormone) pulses frequency, which alter the normal function of the hypothalamus-pituitary-ovarian axis. Furthermore, the ovary dysfunction can be also induced by the prolonged and severe negative energy balance, another consequence of unsuitable management of high yielding dairy cows. The negative energy balance could be worsened by an inadequate feeding strategy with respect to milk yield level. Nevertheless, several other factors (e.g. increased body conditions before calving; unfavourable environmental conditions; health status often related to uterine, hoof and/or metabolic disorders etc.), of different origin as suggested by Formigoni and Trevisi (2003), could be responsible for the marked dry matter intake reduction and, consequently, negative energy balance worsening.

**Immune indices**

Distress has been associated with altered immune function in humans (Kelly et al., 1997) as well as in animals (Salak-Johnson and McGlone, 2007). Both acute and chronic stress tend to affect immune function, but only chronic stress often leads to suppression of the immune system and, as previously underlined, may determine an increase in the occurrence of infectious and metabolic diseases, which in turn cause a reduction in the level of welfare. In addition, the link between chronic stress and immune functions also seems supported by the results obtained in dominant pigs; they seem to have an enhanced immune activation (e.g. greater NK cytotoxicity and phagocytosis and higher leukocyte populations) when challenged with different stressors, whereas subordinates (that are under a psychological stress) show a suppression of the same immune component when submitted to the same stressor (Salak-Johnson and McGlone, 2007).

In agreement with Salak-Johnson and McGlone (2007), the relationship between chronic stress and immune system appears “conflicting and difficult to reconcile into a cohesive and comprehensible set of universally applicable theories”, in particular for the numerous functions of the immune system that have been investigated (both innate and adaptive) and for the discrepancy between the results (Lay and Wilson, 2004). In general, chronic stress leads most often to suppression of some immune functions; in particular, as reviewed by Salak-Johnson and McGlone (2007), the lymphocyte proliferation has decreased in pigs under prenatal stress and social isolation, and in steers, after 3 days of transportation. Other indices, such as Natural killer cytotoxicity, do not show consistent variations; in fact, the activity of Natural killer has been reduced during cold stress in pigs, but increased during other chronic stress (e.g. heat and crowding) (Salak-Johnson and McGlone, 2007). Several immune parameters have been proposed as markers of chronic stress: lymphocyte apoptosis; inhibition of proinflammatory cytokine production; reduction in peripheral mononuclear cells, neutrophil chemotaxis, antibody production; increase in the neutrophil/lymphocyte ratio; functional activity of peripheral blood mononuclear cells.

Within these functions, the immune parameters affected during challenges that have maintained high levels of glucocorticoids in animals seem to be of interest. In fact, as previously discussed, cortisol
seems permanently elevated in conditions of chronic stress. With a high level of glucocorticoids, the number of circulating lymphocytes is reduced, with a lymphopaenia for B, T and natural killer cells and with an eosinopenia; on the contrary the number of neutrophils is increased (neutrophilia). Therefore, in species with relatively low numbers of lymphocytes (e.g. cattle, sheep and pigs), the net result is an increase in the leukocyte count (leukocytosis), while in others species (e.g. chickens) a reduction in circulating leukocytes occurs (Broom, 2006). During chronic stress conditions, the ratio between neutrophils and lymphocytes (N/L) shows a marked rise. This is in agreement with observations by Nemi et al. (1993), who have found levels of the N/L ratio over one in adult cows after prolonged stress, but also during inflammation and endotoxemia, thus suggesting the utility of this ratio in detecting chronic stress. Our results (Bertoni et al., 2003; Trevisi et al., 2003) and those of Amadori (2007) on dairy cows, as well the results of Riondato et al. (2008) on calves, seem to confirm the utility of this index in cattle. Nevertheless, its measurement in the field is quite complex and requires caution because any illness could quickly modify the number and the type of these populations of circulating immune cells in the blood.

Moreover, Kelly et al. (1997) proposed the evaluation of the functional activity of peripheral blood mononuclear cells (PBMC) to assess chronic stress in humans. In fact, the PBMC, incubated with different mitogens which induce non-specific multiplication on lymphocyte, have shown a consistent depression of mitogenesis under chronic stress. In cows, this test has been utilised to demonstrate the impaired situation during heat stress (Lacetera et al., 2006). However, the PBMC test is impracticable for large-scale surveys because the cells must be kept alive and the assays have to be done within few hours after blood sampling. For these reasons, this test is not suitable for large field investigations, but it seems more helpful to validate other simplified methods to assess welfare status.

A further immune indicator to measure welfare status in dairy cows has been suggested by Bonizzi et al. (1989) and Amadori (2007), who found lower levels of the haemolytic complement (a measure of the alternative pathway of the complement defined as properdin) during chronic stress conditions. This alternative pathway of complement activation is able to recognize repetitive sugar structures included in the virus and in the bacteria cell wall; in addition, this pathway activates the classical complement system. Therefore, its reduction could also explain the reduced ability to react against the pathogens.

Biochemical indicators

It is noteworthy that acute stress quickly and transiently determines changes in several blood parameters (e.g. increase in haematocrit, glucose, non esterified fatty acids, creatinine kinase) (table 1); nevertheless, as previously mentioned, acute stress is only rarely linked to welfare. These changes are mainly attributable to endocrine variations, especially of epinephrine, the main “fight or flight” hormone. Contrariwise, some parameters are able to signal persistent consequences or responses, previously defined as maladaptation, and the cause of the chronic stress. Among them the more promising are acute phase proteins and some glycated proteins.

Acute phase proteins. As discussed in the above section, chronic stress can promote a release of pro-inflammatory cytokines: directly (Baugh and Donnelly, 2003) or, more likely, indirectly, as a consequence of the increased susceptibility to the illness (Broom, 2006; Broom and Fraser, 2007), throughout
the reduction of the immune-competence. Disease is, through cytokine release, a cause of welfare reduction (pain, frustration, anorexia, metabolism changes, etc.). However, the release of pro-inflammatory cytokines, whatever the promoting cause might be, triggers the liver to synthesise some proteins usually produced in very low amounts (e.g. haptoglobin, serum amyloid A, ceruloplasmin, protein C reactive, α1-acid glycoprotein etc.) and called positive acute phase proteins (+APP) (Bertoni et al., 1989; Cappa et al., 1989; Gruys et al., 1998; Colditz, 2002; Murata et al., 2004). This physio-pathological answer affects the ordinary functions of liver; namely, it causes the reduction in the synthesis of several other proteins, such as albumin, apolipoproteins, binding proteins of fat-soluble vitamins and hormones (Cappa et al., 1989; Fleck, 1989; Gruys et al., 1998; Murata et al., 2004; Gruys et al., 2005; Bionaz et al., 2007; Bertoni et al., 2008); therefore, they are called negative acute phase proteins (-APP). In this situation, other usual functions of the liver, such as gluconeogenesis (Elsasser et al., 2000), bilirubin excretion (Assenat et al., 2004) and others, can be reduced. Thus, among the mechanisms of the well known negative effects of inflammations, mainly when they are prolonged, the changes in liver functionality could be of great interest and could be useful markers of a chronic stress condition (“disease” stress) in dairy cows (Bertoni, 1999; Bertoni et al., 2008; Lomborg et al., 2008) as well as in beef cattle (Arthington et al., 2003; Arthington et al., 2005) and in pigs (Piñeiro et al., 2007). In particular, the -APP represent promising indicators of chronic stress/reduced welfare during the transition period, as demonstrated in various experiments (Bertoni et al., 2006a; Bionaz et al., 2007; Bertoni et al., 2008), because they are linked to the effects of prolonged and severe inflammation, which cause their marked reduction and reduced level of welfare. In this context, haematocrit could also be a useful index of welfare (Broom, 2003). In fact, chronic inflammatory diseases (Moldawer et al., 1989), as well as a transition period with “heavy” inflammatory conditions, likely as consequences of IL-1, TNFα and cachectin release from macrophages, cause its reduction in the blood for a reduced life span of erythrocytes.

Glycated proteins

The very high level of glucose and/or its frequent increase in the blood, both conditions related to recurrent stresses (mainly the effect of epinephrine and cortisol), have been related to the formation of glycated proteins, for the irreversible bond of glucose or other reducing sugars with proteins. The excessive level of glycated proteins causes some biological detrimental effects, such as inactivation of enzymes, inhibition of the binding of regulatory molecules, inappropriate cross-linking of proteins, slowed removal of abnormal proteins and altered function of genome (Kelly et al., 1997).

For this reason glycated proteins (e.g. glycated haemoglobin and fructosamine) have been investigated as possible markers of chronic stress and, therefore, of a low level of welfare in humans. Glycated haemoglobin (G-Hb) is formed by a slow non enzymatic reaction between glucose and haemoglobin in the red cell and usually shows very low levels in normal subjects (4-6% of the total Hb). In humans G-Hb has shown higher levels in diabetic subjects (Type 1 and Type 2), but also in people exposed to stressful conditions (e.g. psychogenic stressors). In particular, G-Hb concentration is proportional to the time-averaged concentration of blood glucose over the 2-3 months preceding the measurement (Daniel et al., 1999).

Fructosamine (a ketoamine) a derivative of the nonenzymatic reaction product
between glucose and proteins, as well as glycated albumin, have been investigated by Tahara et al. (1995) as potential markers of chronic stress which occurred in previous periods. Fructosamine seems the more interesting because it mimics the glycemia levels in the previous 3 weeks. As with the G-Hb, it is associated with some diseases in humans (e.g. myocardial infarction), but it is independent of G-Hb as demonstrated by their poor correlation (Misciagna et al., 2004), perhaps due to their completely different half-life, because fructosamine includes many proteins and not only albumins.

Unfortunately, to the best of our knowledge, the role of glycated protein levels in relationship to animal welfare conditions has not been investigated yet.

**Other biochemical indices**

Several other parameters have been suggested as useful in detecting chronic stress; among them, two seem to be promising in our experience: potassium and alkaline phosphatase (ALP).

K is an electrolyte, mainly present into the cytoplasm, involved in several cellular processes (e.g. osmotic equilibrium, acid-base equilibrium, regulation of membrane activity). Its level in the plasma is constant and phenomena that could justify its reduction (e.g. fasting, alkalosis, renal dysfunction, use of diuretics) are rare. Hypokalaemia could be caused by some hormones (e.g. insulin and catecholamines), which promote a shift of K⁺ in cells (Halperin and Kamel, 1998). We have, however, signalled a reduction in plasma K during chronic stress, such as relocation of dairy cows from free to tight stalls (Trevisi et al., 1992). This hypokalemic effect seems promoted by hormones related to stress (e.g. oxytocin and epinephrine) which likely cause the increase of K⁺ excretion by kidney.

ALP is an unspecific enzyme able to hydrolyse different types of phosphate esters. In the blood, ALP is detectable in different isoforms as an expression of the organ of origin (bone, liver, kidney, placenta) and could be markedly reduced in some pathological situations (e.g. severe anemia, liver lipodosis, zinc deficiency). During heat stress, mainly in primiparous cows, Wazhapilly et al. (1992) have signaled a marked reduction in ALP and this result was later confirmed by Abeni et al. (2007). Furthermore, a decrease in ALP has also been signalled in other stressful conditions, such as changing of groups and high stocking density in dairy cows (Calamari et al., 2003) or as a consequence of social stress in pigs (Tuchscherer et al., 1998).

In any case, both these indices (K and ALP) need more research to be confirmed as reliable markers of chronic stress.

**Conclusions**

Animals do not perceive necessary stressful events as negative experiences; this situation can be defined as eustress and it occurs when animals are able to successfully cope with the challenge and to adopt effective strategies of adaptation. Under these conditions, stresses are often well tolerated, despite the fact that they induce important physiological (mainly endocrine and metabolic) variations, which are transient and, therefore, this kind of stress is defined as “acute”.

For their welfare implications, chronic stress conditions are more interesting. They are less evident but long term changes in endocrine, physiological and immune functions occur with pre-pathological or pathological consequences. The evaluation of this kind of stress could therefore be interesting and to carry out such an evaluation several indicators have been proposed (e.g. cortisol, ACTH challenge, heart rate variability, acute phase proteins, neutrophils lym-
phocytes ratio, glycated haemoglobin, etc.). Unfortunately many of them are not useful due to their low sensitivity, analytical difficulties and scarcity of research.

Among them, the most used index of the chronic stress remains the cortisol, despite the interpretation of its basal level and of its variations after ACTH challenge are still in discussion. In fact, high basal level of cortisol in plasma likely reflects a chronic stress status in cows, but the correct measurement is difficult for the strong sampling effect. The evaluation of cortisol in alternative body compartments (e.g. saliva, milk, and mainly faeces) could represent a possible solution, but again their standardisation needs more investigations. Perhaps, the efforts to evaluate the reactivity of adrenal gland (e.g. ACTH or CRH challenges) require more attention because they could improve the knowledge of the stress status. Namely, a better standardisation of the these challenge-tests seems urgent to compare the results obtained from different studies.

Among the indices to consider (e.g. endocrinological, physiological, performance, health, immunological, biochemical), the most useful in field conditions are the performance and health indicators. Performance indices provide a measure of maladaptation and, therefore, of the welfare status. Some of them (e.g. milk yield and fertility) are usually monitored on dairy farms; therefore, their availability is inexpensive and, moreover, they also allow a retrospective analysis of chronic stress. The health conditions could also be useful indicators of chronic stress, especially if each case of disease or disorder is routinely recorded. Moreover, other markers of chronic stress could be easily utilised in the field, better if in association with classical indicators (e.g. cortisol), such as negative acute phase proteins, neutrophil/lymphocytes ratio, haemolitic complement. On the other hand, the link with the chronic stress need more investigations for several parameters: some easily detectable (e.g. glycated proteins, ALP, K) and other difficult to be evaluated (e.g. β-endorphins, heart rate variability, functional evaluation of peripheral blood mononuclear cells).

In conclusion, the objective detection of chronic stress remains an essential goal in the livestock breeding field. In fact, the assessment of animal welfare, which includes some chronic stress parameters, can be utilised for the optimisation of livestock production which means healthy and content producing animals. Finally, the complexity of the biology of stress requires more extensive and in-depth studies to identify the best markers of chronic stress that would be useful in field conditions.

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