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TESI DI DOTTORATO DI RICERCA

RELATIONSHIP BETWEEN GENETIC MERIT AND BIOLOGICAL RESPONSE IN LACTATING COWS

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Executive summary

The research reported in this thesis refers to two studies, that were conducted in commercial farms, and the results were arranged in two parts.

Part 1 deals with the association between estimated breeding values (EBVs) and hematological, milk and urine markers of metabolism and well being of dairy cows after the peak of lactation. The study 1 used 153 lactating cows, selected from 3 Simmental (IS) and 2 Holstein (IH) commercial farms. Cows were ranked according to the EBVp from minus to positive and selected every 5 EBVp values from minus to positive. Milk samples were individually collected and analysed for protein, fat, lactose, β-hydroxybutirate (BOHB), cortisol contents and somatic cell count (SCC). Blood was sampled after the morning milking and before the morning meal, and urines were sampled after stimulation of micturition. Blood samples were analysed for total protein, albumin, globulin, urea, glucose, creatinine, aspartate aminotransferase (AST), haemoglobin (Hb), non-esterified fatty acids, BOHB, glutathione peroxidase (GPx), total antioxidant status (TAS), cortisol and Zn, and urine for total N, urea and purine derivatives (PD) N. Significantly higher milk ($P < 0.001$) and fat ($P < 0.01$) yields and significantly lower body condition score (BCS), protein percentage, SCC, urea and BOHB ($P < 0.001$) were observed for IH cows in comparison to IS. Significant differences were observed for plasma creatinine ($P < 0.001$), higher in IS, and for AST ($P < 0.05$), Zn, TAS and GPx ($P < 0.001$), higher in IH cows. The concentrations of N ($P < 0.01$), creatinine N and the creatinine N to N ratio ($P < 0.001$) in urine were significantly higher in IS than IH cows. Instead, the PD N to total N ($P < 0.05$) and PD N to creatinine N ratio ($P < 0.001$) were lower in IS than in IH cows. A positive linear effect of EBVp was observed for milk yield, milk protein and fat yields ($P < 0.05$) for IS and IH cows. The EBVp was negatively related to BCS and glucose in IS cows ($P < 0.05$) and to plasma BOHB in both breeds ($P < 0.05$). EBVp was negatively related ($P < 0.05$) to urinary urea to total N and PD N to total N ratios in IS cows and to PD N to creatinine N ratio for IH cows.

Biomarkers of metabolism measured in blood, urine and milk, often used to assess the energy and nitrogen balance during peripartum, can also be used to understand differences related to the breed and to the genetic merit.

Study 2 was conducted on 10 commercial farms, 6 of IS and 4 of IH, for a total number of 1200 lactating cows. For these animals, EBV for milk fat (EBVf) and protein (EBVp) yields and for somatic cell count (EBVc) were associated with markers of metabolism and well being in blood and with cortisol in milk and hair. The results confirmed the negative association of BOHB with EBVp ($P < 0.01$) for both breeds and also a positive linear association with EBVf ($P < 0.01$). Differences between breeds were also confirmed for plasma creatinine, that was significantly higher ($P < 0.01$) for IS than IH. However, cortisol in hair and milk was not related to EBVs.

In part 2, cows were grouped on the base of SSC (class1 < 200,000/ml; class 2 200001 < SCC < 400000; class 3 > 4000000). The aim was to investigate the relationship between cortisol concentrations, in milk, blood or hair, and the SCC, considering also the effect of farm, milk yield and days in milking (DIM).

For study 1, a total of 135 cows were sampled from 2 commercial farms of Italian Simmental (IS) cows and 2 commercial farms of Italian Holstein (IH), whilst in study 2, a total of 1041 cows were sampled from 6 commercial farms of IS and 4 commercial farms of IH.

In study 1, the values of cortisol content in milk were higher in IH than IS cows. Significant effect ($P < 0.01$) was shown between farms for milk and plasma cortisol concentrations. Cortisol content in milk was not correlated to plasma content in study 1 and the mean milk to plasma cortisol ratio was about 1:30. In study 2, significantly higher values of milk cortisol for IH cows in comparison to IS cows were reported ($P < 0.01$). A significant effect of class of SCC was observed, cows belonging to class 3 showed significant higher milk cortisol content. The mean values of milk cortisol were 6.15 for class 1, 6.14 for class 2 and 6.27 for class 3.

Acute phase proteins in plasma significantly differ for class of SCC. Haptoglobin and ceruloplsmin in plasma were significantly higher ($P < 0.05$) for class 3 in comparison to class 2

and to class 1. Paroxonase and albumin were signicantly lower ($P < 0.05$) in class 3 than in the other classes of SCC. The farm effect was significant also in the study 2, confirming the results obtained in the first study.

Biomarkers of metabolism and oxidative stress were also in line with the EBVs and in particular BOHB is a promising compound to measure also in mid lactation, when subclinical ketosis is not more a potential problems for the lactating cows, since contains genetic information other than nutrition relationship.

Instead, biomarkers of animal health, as acute phase proteins in plasma and cortisol in plasma, milk or hair, are more related to environment and, probably, the complex regulatory mechanisms of these compounds does not allow to link them directly to genetic background. These biomarkers, and milk cortisol in particular, are promising compound to measure to complement the assessment of animal welfare protocols.

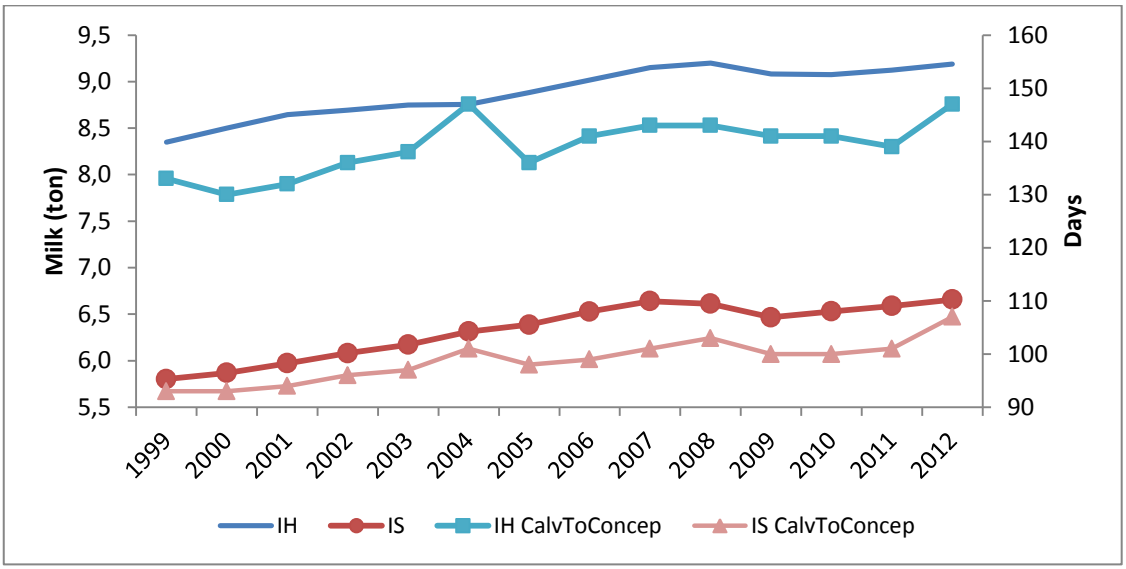
Key words: metabolism, biomarkers, genetic merit, milk cortisol, hair cortisol, cows

Introduction

1.Setting the scene

The selection of dairy cows was mainly based in the past to improve productive traits, as milk yield and fat and protein content (NN), and among the functional traits, the somatic cell content. As a consequence, a dramatic increase in milk yield per cow was observed in the last 20 years due to the rapid progress in genetic and management (Figure 1).

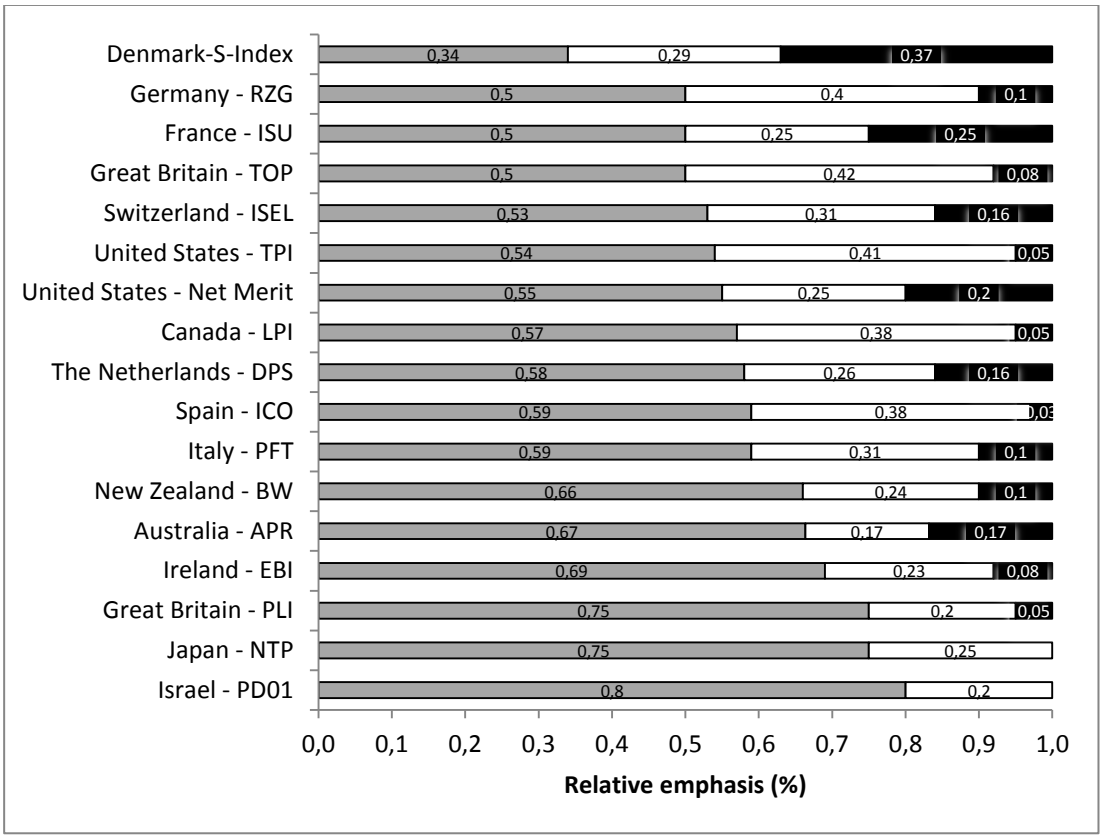
Figure 1. Trend of the productive and reproductive performances of the Italian Holstein (IH) and the Italian Simmental (IS) dairy cattle across last 13 years. Milk yield (left axis) refers to the standardized 305d lactation and CalvToConcep (right axis) refers to the number of days from calving to conception.



Source: AIA

More recently, international breeding goals in dairy cows included also functional traits such as fertility, udder health, type traits and functional survival (Figure 2). The main reasons for this shift are the European quota-based milk marketing system and price constraints, together with increasing producer and consumer concerns associated with the observed deterioration of the health and reproduction of dairy cows (Miglior et al., 2005).

Figure 2: Relative emphasis on longevity (grey), durability (white), and health and reproduction (black) components in Holstein selection indexes of countries in August 2003 (from Miglior 2005). More recent data are lacking due to the Interbull company property and secrecy policy. Acronyms close to the countries indicate the respective national selection index.



The current selection index for the Italian Holstein breed is the PFT (Productivity, Functionality and Type) (http://www.anafi.it/). It combines milk quality and functionality to determine a 49:51 ratio between the weights of the productive and functional traits (Table 1).

The current selection index for the Italian Simmental breed is the IDA (Dual purpose index) (http://www.anapri.eu/). This breed is selected to produce both milk and beef, although the dairy traits has become prevalent. Thereof in the selecting index, together with traits related to milk production and functionality, aspects related to the production of meat are considered (Table 2).

Table 1. The Italian Holstein selection index, as published by the Italian Association of Frisona Italiana breeders (ANAFI).

PRODUCTIVITY		FUNCTIONALITY	
Trait	Weight	Trait	Weight
Milk yield	0	TYPE	4
Fat yield	8	ICM	13
Protein yield	36	IAP	6
Fat %	2	Somatic cells	10
Protein %	3	Functional longevity	8
		Fertility	10

TYPE: index derived from 15 defined linear traits; ICM: Udder composite index; IAP: Feet & Legs composite index

Table 2. The Italian Simmental selection index, as published by the Italian Association of Pezzata Rossa Italiana breeders (ANAPRI)

MILK PRODUCTION		BEEF PRODUCTION		FITNESS		FUNCTIONALITY	
Trait	Weight	Trait	Weight	Trait	Weight	Trait	Weight
Protein kg	5	PT beef index	18	Udder	14.5	Milkability	7.5
Fat %	2	Cow muscularity	6	Feet & Legs	5	Somatic cells	-5
Protein %	5						

PT: performance test of the young bulls

The genetic merit for milk yield and composition of dairy cows is estimated from phenotypic data registered in the farms during the official records and with the application of appropriated statistical models (Pritchard *et al.*, 2013). Before the advent of genomic selection, the genetic progress of animals was largely dependent from the results derived from the statistical models elaborated by quantitative genetic, which define the estimated breeding values (EBV) for each of the recorded trait (Hayes *et al.*, 2009). The continuous selection based on the EBV and the collaboration among Countries has led to the progress of the productive traits, as milk yield and its constituents. The associated variations of digestive and metabolic efficiencies of the dairy cows under selective pressure is a fascinating field of research (Kelly *et al.*, 2011; Cassandro *et al.*, 2013) that requires further investigations.

Ruminants provide an indispensable source of nutrients for consumption in the planet and their number in the world is 3.45 billions, 1.53 cattle and buffalos and 1.92 sheep and goats (FAO, 2012). The estimated world allowances of milk is 680 millions of tons but, according to FAO (2012) and related estimates, the trend for the demand of milk is doubling in the next 20 years, due to a growing consumption of dairy products in Asia, including China, and South America. Livestock production and dairy farming in particular have to face with this future scenario. Expanding animal productions, to cope with the exponential growth of human population and food demands, will require an increase of land use for crop and pasture, which is in contrast with its utilization for non-agricultural purposes and the international agreements to stop the erosion of natural resources (e.g. forests). With this perspective, the growing competition between humans and livestock for land and energy use and the increased environmental impact of farming systems, also affecting climate changes, require new directions and strategies for animal productions.

These aspects strongly call for an increased efficiency of dairy cattle production, to enhance the environmental sustainability of this livestock system, still satisfying the human demand for safe milk and dairy products. Efficient and rationale use of resources, increasing the efficiency and

feeding utilizations and reducing wastes, are of paramount relevance for the farm of the future and can be view as a strategic agenda which also involve animal breeding and husbandry. In addition, Citizens, Experts, Scientists and Politics have raised many concerns on animal welfare, in its broader terms, and the challenge to ensure better quality of life for livestock has jumped over the catwalk, gaining attention and opening ethical, scientific and technical debates.

It is generally accepted from citizens that high input systems do not take enough into account animal priorities, looking more to exploitation of resources and to the economy of the farm. Apparently, livestock efficiency and animal welfare are in contrast, although there are growing convincing evidences that demonstrate how cows farmed in more suitable environmental conditions can improve efficiency, since the costs to face with unpleasant and uncomfortable situations redirect bodily resources towards catabolism and reduce anabolism (Elasser et al., 2000). For the above reported reasons, livestock systems will focus to more environmental sustainable productions and requires more efficient animal kept in an optimal welfare. These main drivers of future dairy cattle farming will rule the breeding schemes of tomorrow and the identification and the recording of new phenotypes, which depict animal efficiency and welfare, will be required.

2. Phenotype for animal efficiency

Feed efficiency can be calculated in many different ways, all requiring accurate measures of dry matter and nutrient intake. Recently, Berry and Crowley (2013) comprehensively reviewed alternative ways of calculating feed efficiency, which they defined as being categorized into: (1) traits that are ratios and (2) residual or regression traits.

Ruminants produce enteric methane as part of their fermentation processes, and the accumulation of methane in the earth's atmosphere is linked to climate change. Enteric methane energy output per unit gross energy intake or milk energy output is negatively related to levels of milk

production, energy content of the diet and the efficiency of metabolisable energy utilisation for lactation.

The methane losses are mainly related to digestive process and fiber fermentation account for a large part of its emission. After digestion and absorption, nutrients are utilized by the body according to priorities and with different efficiency, which also varies among individuals.

A method to describe animal efficiency is the so call "Residual feed intake" (RFI), sometimes referred to as net feed intake, that is the difference between actual and predicted DMI (Koch et al., 1963; Berry and Crowley, 2013). RFI is calculated as the residual from a regression model of DMI on various energy sinks, such as milk production and live weight (for maintenance requirements). Additional requirements such as energy required for activity RFI is of importance because it conceptually captures variation in activity, protein turnover, digestibility and heat increment of fermentation (Herd and Artur, 2009) and, as such, is often referred to as a measure of metabolic efficiency. The RFI trait is moderately heritable and genetically independent of growth and body size (Crews, 2005). However, knowledge of the underlying biological mechanisms controlling RFI are yet to be fully understood (Herd and Arthur, 2009; Moore et al., 2009). Basal metabolic rate is associated with cellular processes, as protein turnover and mitochondrial function, and accounts for the largest fraction of total energy expenditure and for the large majority of inter-animal variations. A recent research (Kelly et al., 2011) has examined these processes in productive cattle, providing evidences of association between mitochondrial biogenesis and energetic efficiency and suggesting that the expression of some genes and their transcriptional regulators may provide potential indicators for genetic variation in feed efficiency.

There is limited data available on genetic correlations between RFI and fertility. In beef, Johnston et al. (2009) reported indirect evidence of a negative genetic correlation (−0.6) between fertility (age at puberty) in heifers and RFI measured in their paternal half-brothers for Brahman, but not for Brahman crosses (where the genetic correlation is 0.02). Vallimont et al. (2012) used

a data set of 970 Holsteins and found unfavorable genetic correlations between RFI and three measures of fertility (daughter pregnancy rate, cow conception rate and heifer conception rate). Again low RFI (i.e. efficient) cows have poorer fertility.

Selection for RFI has been reported not only to increase metabolic efficiency but also to reduce methane emissions (Hegarty et al., 2007). Similar results were reported by Nkrumah et al. (2006), which estimated in steers a phenotypic correlation of 0.44 ($P < 0.05$) between RFI and methane production. In dairy cattle, De Haas et al. (2011) suggested that by selecting for more efficient cows, methane production could be reduced by up to 26% over a 10-year time folds.

The assessment of digestive and metabolic efficiencies is very complex, considering the countless physiological factors affecting these processes, and biomarkers can represent an alternative approach to investigate these aspects. Non-esterified fatty acids (NEFA) concentration, β-hydroxybutyrate - BOHB and urea in blood, BOHB and urea in milk (Kelly et al., 2010), total purine derivatives (PD) excretion (Stefanon et al., 2001) or PD to creatinine ratios (Susmel et al., 1995) in blood and urine have been already used as biomarkers of metabolism and feeding efficiency.

The concentration of BOHB in blood and in milk is generally used to diagnose ketosis in lactating cows, one of the most common disease in high producing dairy cows. Ketosis is caused by a negative energy balance and typically occurs within 2 month after calving. Clinical and subclinical ketosis both result in increased concentrations of ketone bodies in tissues and milk of the cows. Blood BOHB concentration has often used for this detection, and several authors used a cut-off point of 1200 μmol/L to discriminate between healthy cows and animals with subclinical ketosis (Geishauser et al., 1998; Jorritsma et al., 1998). Even when clinical signs do not appear, ketosis can affect milk production (Gustafsson et al., 1993) and reproduction (Andersson et al., 1991) and is associated with an increased frequency of left displaced abomasum (Geishauser et al., 1997) or a decreased of non specific immunity (Sartorelli et al., 2000).

Recent studies has indicated that BOHB can be related not only to ketosis during the transition period of lactating cows, but can also be used as index of metabolic efficiency in steers.

Although this biomarkers is not validated yet and no relationship has been established with RFI in lactating cows, the hereditability of milk BOHB, ranging from 0.13 to 0.29 (Koeck et al., 2014), would indicated that its determination in biological fluid can be used not only for diagnostic purposes but also for genetic improvement.

The description of the new phenotypes is completed with productive data, as milk yield, milk quality and hygiene and body condition score (BCS).

Correlation between stress response and cortisol secretion and metabolic efficiency of cattle, measured as residual feed intake (RFI), was recently investigated. In beef steers, evidences for genetic associations for RFI with plasma cortisol and blood cell variables have been published, indicating that animals with high-RFI (low efficiency) are more susceptible to stress (Richardson et al., 2004).

Stress modifies the secretion of various hormones, the immune system and blood constituents with consequences that depend on the type of stimulus, the species, the sex, and the individual considered (Amadori et al., 2009). Among the stress responsive hormones, glucocorticoids play an important role in shaping immunity by influencing immune cell trafficking to sites of inflammation and altering downstream, adaptive immune responses by causing a shift from cellular (Th1/inflammatory) to humoral (Th2/anti-inflammatory) type immune responses (Elenkov and Chrousos, 1999). Moreover, activated macrophages, endothelial cells, lymphocytes and other immunity related cells induce the synthesis of pro-inflammatory cytokines, which stimulate the liver to synthesize positive acute phase proteins (APP+), such as haptoglobin and ceruloplasmin (Bionaz et al., 2007). Another important consequence of animal response to stress can be the increase of oxidative metabolism of glucose and lipids. Cortisol diverts body resources to the blood stream to face with the increasing demand of energy and protein substrates required to respond to the stressors, reducing the overall efficiency of the animal.

The two phenotypes are part of the same adaptative response of the animal to the environment. It is likely that only a system biology approach will help to identify the factors driving the productivity of the dairy cows, that can be associated to genotypes and integrated in genomic selection.

Stress resistance (SR) and animal efficiency (as RFI) are thus strongly related and can be measured with indirect biomarkers, which can describe these new phenotypes. As already reported, for the SR phenotype, the gold standard of biomarkers is cortisol, which can be measured in milk, blood, hair and feces. Instead, RFI is a complex trait and more than a single biomarker is probably required for its description. The selection of dairy cows with a higher adaptation to environmental and physiological stresses positively affect also animal efficiency, decreasing the negative effects of immune suppression and enhancing the quality of life of the animals in the farm. It is plausible that, for the above reported reasons, a genetic correlation among RFI and other traits would exist and, if true, it will be interesting for selection purpose to include RFI in breeding programs. However, dry matter, intake, diet composition, body weight and body weight change of the animal, other than milk yield and composition are required for the determination of RFI, and some of these variables are not easily recorded in commercial farms.

3. Endocrinology of stress response

The stress response is an evolutionarily conserved process mediated by the hypothalamic – pituitary–adrenal (HPA) axis that allows organisms to respond rapidly to unpredictable changes in their environment.The physiological response to short-term stress is adaptive (‘‘fight or flight’’), while long-term stress (occurring over weeks to months) may lead to pathological syndromes of distress characterized by immunosuppression, decreased reproduction, and diminished growth (Moberg, 2000; Habib et al., 2001; Charmandari et al., 2005).

The endocrinology of stress response recognizes both central nervous system (CNS) and peripheral components (Habib et al., 2001; Chrousos, 2002). The central components of the stress system are located in the hypothalamus and the brainstem, and include:

a) the parvocellular neurons of corticotropin-releasing hormone (CRH);
b) the arginine vasopressin (AVP) neurons of the paraventricular nuclei (PVN) of the hypothalamus;
c) the CRH neurons of the paragigantocellular and parabranchial;
d) nuclei of the medulla and the locus ceruleus (LC);
e) other mostly noradrenergic (NE) cell groups in the medulla and pons (LC/NE system).

The peripheral components of the stress system include:

a) the peripheral limbs of the hypothalamic-pituitary-adrenal (HPA) axis;
b) the efferent sympathetic-adrenomedullary system;
c) the components of the parasympathetic system.

Reciprocal reverberatory neural connections exist between the CRH and noradrenergic neurons of the central stress system, with CRH and norepinephrine stimulating each other primarily through CRH type 1 and α1-noradrenergic receptors, respectively. Negative feedback loops, which are autoregulatory, are also present in both the PVN, CRH and brainstem noradrenergic neurons (Silverman, 1989) with collateral fibers inhibiting CRH and catecholamine secretion via presynaptic CRH and α2-noradrenergic receptors, respectively (Aghajanian, 1982). Both the CRH and the noradrenergic neurons also receive stimulatory innervation from the serotoninergic and cholinergic systems (Fuller, 1996), leading to inhibition of inputs from the γ -aminobutyric acid (GABA), benzodiazepine (BZD) and opioid peptide neuronal systems of the brain as well as

from the end-product of the HPA axis, the glucocorticoids, GC, (Keller-Wood and Dallman, 1984).

The CRH is the main hypothalamic regulator of the pituitary-adrenal axis, and together with its receptors, is found in many extra hypothalamic sites, including limbic system, basal forebrain, LC-NE sympathetic system in the brainstem and spinal cord.

In mammals, family members include CRH, urocortins (UcnI, UcnII and UcnIII), CRHR1 and CRHR2 receptors and a CRH-binding protein. These family members differ in their tissue distribution and pharmacology and play an important role in the regulation of the endocrine and behavioral responses to stress. CRHR1 and CRHR2 are products of distinct genes and have several splice variants expressed in several central and peripheral tissues.

The CRHR1 subtype is widely distributed in brain, mainly in the anterior pituitary, the neocortex and the cerebellum, adrenal gland, skin, ovary and testis (Charmandari et al., 2005). CRH-R2 receptors are expressed mostly in peripheral vasculature, skeletal muscles, gastrointestinal tract and heart, but it is also found in subcortical structures of the brain, such as the lateral septum, amygdala, hypothalamus and brain system.

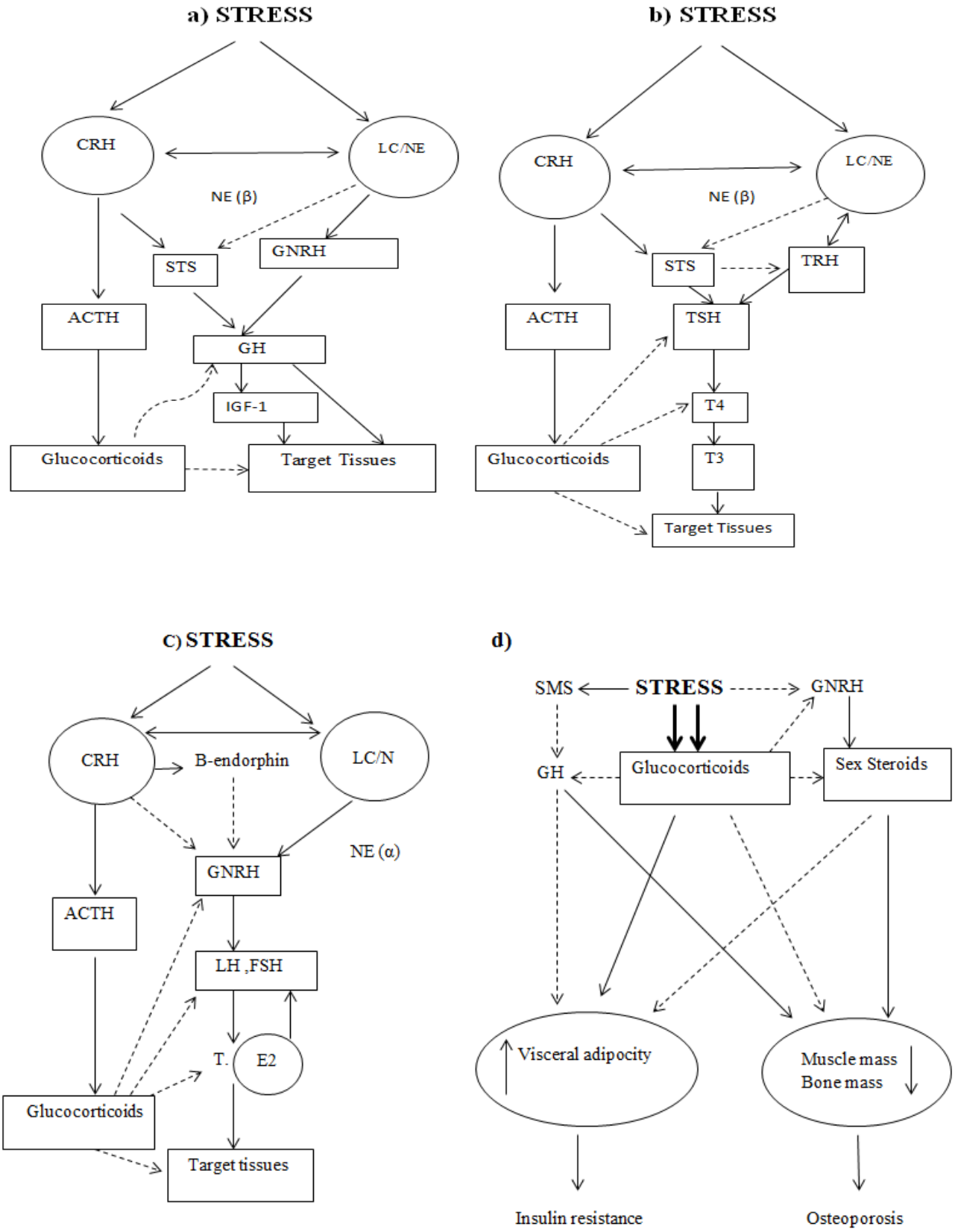
CRH is the main hypothalamic regulator of the pituitary-adrenal axis and stimulates the secretion of adrenocorticotropin hormone (ACTH) from the anterior pituitary. A positive reciprocal interaction between CRH and AVP also exists at the level of hypothalamus and each neuropeptide stimulates the secretion of the other.

In non-stressed humans, both CRH and AVP are secreted in the portal system in a circadian, pulsatile and highly concordant fashion.In man, the amplitude of the CRH and AVP pulses increases early in the morning, resulting in increases primarily in the amplitude of the pulsatile ACTH and cortisol secretion. In cattle, the circadian rhythm of cortisol is very week, instead an ultradian pulsatile secretion with an oscillation of around 120 minutes has been reported (Lefcourt et al., 1993).

Diurnal variations in the pulsatile secretion of ACTH and cortisol are often perturbed by changes in lighting, feeding schedules, and activity, as well as following stress. During acute stress, there is an increase in the amplitude and synchronization of the PVN, CRH and AVP pulsatile release into the hypophyseal portal system. In addition, depending on the stressor, other factors, such as angiotensin II, several cytokines and lipid mediators of inflammation are secreted and act on the hypothalamic, pituitary, and/or adrenal components of the HPA axis and potentiate its activity.

The adrenal cortex is the main target of ACTH, which regulates GC and adrenal androgen secretion by the *zona fasciculata* and *reticularis*, respectively, and participates in the control of aldosterone secretion by the *zona glomerulosa*. Other hormones, as cytokines and neuronal information from the autonomic nerves of the adrenal cortex, can also be involved in the control of cortisol secretion.

Figure 3. Schematic representation of the interactions between the stress system and (a) the GH/IGF-I axis, (b) the thyroid axis, (c) the hypothalamic-pituitary gonadal axis, and (d) metabolic functions (from: Charmandari et al., 2005)



Prolonged activation of the HPA axis leads to suppression of growth hormone (GH) secretion, and the increased release of GC inhibits the effects of insulin-like growth factor I (IGF-I) and other growth factors on target tissues (Figure 3a). The secretion of CRH and of GCs increase somatostatin secretion and inhibit GH secretion, which are implicated as a potential mechanism of chronic stress-related suppression of GH secretion (Charmandari et al., 2005). However, acute elevations of serum GH concentrations may occur at the onset of the stress response or following acute administration of GCs, most likely due to stimulation of the GH gene by GCs through glucocorticoid responsive elements (GREs) in the promoter region of the gene (Raza et al., 1998).

Thyroid function is also inhibited during stress (Figure 3b) and the activation of the HPA axis is associated with a decreased production of thyroid-stimulating hormone (TSH) and with an inhibition of peripheral conversion of the relatively inactive thyroxine to the biologically active triiodothyronine (Benker et al., 1990). These modifications may be due to an increase of somatostatin concentrations following the enhanced secretion of CRH and GCs. Somatostatin suppresses both TRH and TSH, whereas GCs inhibit the activity of the enzyme 5-deiodinase, which converts thyroxine to triiodothyronine.

The activation of inflammatory cascade increase the secretion of cytokines, such as TNF-α, IL-1 and IL-6, which in turns activate CRH secretion and inhibit 5-deiodinase activity (Chrousos, 1997). The reproductive axis is inhibited at all levels by various components of the HPA axis (Figure 3c) and CRH suppresses the secretion of gonadotropin-releasing hormone (GnRH), either directly or indirectly, by stimulating the arcuate POMC peptide secreting neurons (Rivier et al., 1986; Vamvakopoulos et al., 1994). GCs also exert an inhibitory effect on the GnRH neuron, the pituitary gonadotroph and the gonads and render target tissues of gonadal steroids resistant to these hormones (Rivier et al., 1986; Mac Adams et al., 1986).

The interaction between CRH and the hypothalamic-pituitary-gonadal axis is bidirectional, since estrogen increases CRH gene expression via estrogen response elements (EREs) in the promoter

region of the CRH gene (Vamvakopoulos et al., 1993). The CRH gene is also a target of gonadal steroids and, potentially, mediates sex-related differences in stress-response and activity of the HPA axis. In addition to their direct catabolic effects, GCs promote adipogenesis since antagonize the actions of GH and sex steroids on fat tissue catabolism (Figure 3d) and muscle and bone anabolism (Chrousos, 2002). Chronic activation of the stress system is associated with increased visceral adiposity, decreased lean body (bone and muscle) mass, and suppressed osteoblastic activity, a phenotype observed in patients with Cushing's syndrome. Progressive glucocorticoid-induced visceral adiposity causes further insulin resistance and deterioration of the glycemic control. Therefore, chronic activation of the HPA system in patients with diabetes mellitus may result in a vicious cycle of hyperglycemia, hyperlipidemia, and progressively increasing insulin resistance and insulin requirements. A decrease of turnover in osteoporosis is consistently reported in association with hypercortisolism and GH deficiency, representing a further example of the adverse effects of chronic elevated cortisol concentrations and decreased GH/IGF-I secretions on osteoblastic activity. The stress-induced hypogonadism and the reduced concentrations of sex steroids may further contribute to the development of osteoporosis.

The GC system includes also the nuclear GC receptor (GR) and is found in all vertebrates, consistently with an evolutionary well-conserved mechanism of stress response. The fine GC mechanism assists the organism to regulate cell growth, bone density, metabolism and cardiovascular system, other than influencing behavior (Charmandari et al., 2005). There is considerable individual variations in sensitivity to GCs (Hearing et al., 1999), which may affect the outcome of GC stimulations by environments and treatments.

Mutations and splice variants of the GR gene lead to the subsequent polymorphisms of the protein in humans and other vertebrates. The variability of translated proteins, rather than an alteration in the consensus sequence of the GR-binding site in the DNA of target genes, may explain the individual variation in individual sensitivity to GC (Bray and Cotton 2003; Stevens et al. 2004; Van Rossum et al., 2002).

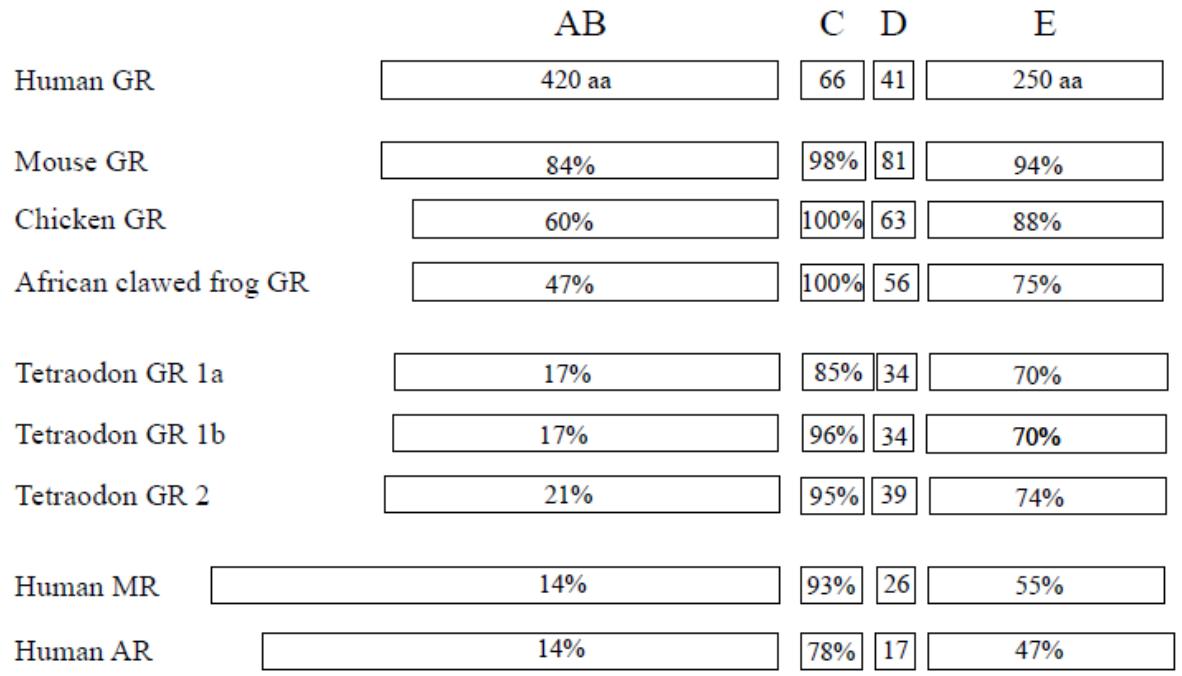
Many studies have demonstrated in a variety of systems that there is a direct correlation between the concentration of the GR in a cell and its sensitivity to GCs. Genetic abnormalities of the GR are related to mutations which inactivates the ligand binding domain or leads to functional knockout of one of the two GR gene alleles. These mutations lead to a generalized GC resistance, compensated for by hyperactivity of the HPA axis. The GR (Hollenberg et al., 1985) structure and function is well established in mammals and conserved among known vertebrate species. The N-terminal region varies greatly in size and composition among different members of the superfamily and is involved in transactivation of downstream genes. Mutations in this domain decrease transcriptional activity of target genes without affecting ligand affinity (Giguere et al., 1986). The DNA-binding region is the central domain and binds to GRE in the promoter regions to initiate transcription of GC responsive genes. The amino acid sequence of this region is strictly conserved, both in different members of the superfamily and virtually in all vertebrate species (Figure 4).

The hinge region, involved in conformational changes during receptor–ligand binding, is quite variable in its length and amino acid sequence. The relatively well-conserved ligand binding region is situated at the C-terminus (Stolte et al., 2006). The marked similarity of amino acid sequences in the ligand binding region explains why multiple receptors can bind the same ligand and, subsequently, elicit a similar response of a downstream gene.

In the absence of ligand, the non-activated glucocorticoid receptor (GR) resides primarily in the cytoplasm of cells as part of a large multiprotein complex consisting of the receptor polypeptide, two molecules of HSP90 and several other proteins (Bamberger et al., 1996). Upon hormone binding, the receptor dissociates from HSP90 and other proteins, translocates into the nucleus, where it binds as homodimer to GREs located in the promoter region of target genes, and up or down regulates the expression of glucocorticoid-responsive genes, depending on the GRE sequence and promoter context. The receptor can also modulate gene expression independently of GRE-binding, by physically interacting with other transcription factors, such as activating

protein-1 (AP-1) and nuclear factor-κB (NF-κB) (Bamberger et al., 1996). A dual-receptor system exists for glucocorticoids in the CNS, which includes the GR type I or mineralocorticoid receptor that responds to low concentrations of glucocorticoids, and the classic GR type II that responds to both basal and stress concentrations of glucocorticoids. The negative feedback control of the CRH and ACTH secretion is mediated through Gr type II. A genetically determined imbalance of the glucocorticoid receptor isoforms was found recently in cultured lymphocytes from a patient with congenital generalized glucocorticoid resistance and chronic leukemia (Magiakou et al., 1994).

Figure 4. Percentage amino acid identity of the different receptor regions of several members of the nuclear receptor superfamily of vertebrate species. Length of the boxes represents lengths of particular regions. MR, mineralocorticoid receptor; AR, androgen receptor. AB, N-terminal region; C, DNA-binding region; D, hinge region; E, ligand binding region (from Stolte et al., 2006).



4. Phenotype of animal welfare

The stress response of an individual is determined by multiple factors. We have reported that genetic polymorphisms, such as those of CRH, AVP, and their receptors and/or regulators, account for the observed variability in the function of the stress system. This genetic vulnerability is polygenic by nature and allows a modulation of phenotype in the presence of environmental triggers. There is a complex genetic background continuum in animal population that ranges from extreme resilience to extreme vulnerability to the same stress-related comorbid states (Figure 5). Stressors of gradually decreasing intensity may be sufficient to discriminate individuals, whose genetic vulnerability places them on the vulnerable side of the continuum (Charmandari et al., 2005).

The dose-response relationship between the intensity of the stressor and the level of responsiveness of the biological system can be represented by a sigmoidal curve, which is expected to differ from individual to individual. The dose-response curve can be shifted to the left of to the right of that of an average reactive individual (Figure 6). The latter denotes an excessive reaction, whereas the former a defective one. Similarly, the dose response relationship between the individual feeling of well-being or individual performance ability with the response of the stress system can be represented by an inverted U-shaped curve that covers the range of the activity of the latter. Shifts to the left or to the right of this range would result in hypo arousal or hyper arousal, respectively, and a suboptimal sense of well-being or diminished performance (Chrousos, 1999).

Positive influences during development shift an individual toward a more resilient response to stress, whereas if negative have the opposite effect (Figure 7). Therefore, a supportive or an adverse environment interfere with the occurrence of one or more of the above stress-related states, indicating that genetic and development define vulnerability, whereas environment determines the triggering and/or severity of a disease.

In a broader sense, resilience and vulnerability in dairy animals can be associated to the concept of robustness, a definition to describe 'the ability to maintain homeostasis in commonly accepted and sustainable dairy herds in the near future' (Ten Napel et al., 2009). The term robustness is generally used in relation to non-productive or so-called functional traits, and is defined as the ability of the individual to effectively respond to stressors. In the last few decades, dairy cattle breeding goals have broadened from production traits to production and functional traits in a balanced breeding goal (Miglior et al., 2005; Shook, 2006). Nevertheless, most of these traits are either indicator traits or index traits for other underlying traits, which are usually difficult or expensive to measure in a sufficiently large population to undertake accurate genetic evaluations. Each of these indicator traits are complex phenotypes of underlying physiological phenotypes. New low-cost phenotyping strategies are currently under investigation to better predict or measure these phenotypes associated with animal robustness traits.

Dairy animals experience a large variety of stressors that can modify normal behavior and growth, leading to losses in performance (Amadori et al., 2009). Normal physiological events such as calving, milk yield, weaning and group rearrangement can cause metabolic and environmental conditions which lead to stress, a consequent impairment and loss of animal welfare (Lykkesfeldt and Svendsen, 2006) and a decrease of safety and quality of products. Under these stressful conditions, the HPA axis, the autonomic nervous system and the immune system are called into action to re-establish homeostasis (Amadori et al., 2009). Stress modifies the secretion of various hormones (Amadori et al., 2009) and among these glucocorticoids play an important role in shaping immunity by influencing immune cell trafficking and causing a shift from cellular to humoral type immune responses (Elenkov and Chrousos, 1999).

Figure 5. Representation of the genetic continuum that defines the genetically determined vulnerability/resilience to stressors of an individual. Vertical arrows indicate the extent of environmental stressors which can lead to disease (Charmandari et al., 2005).

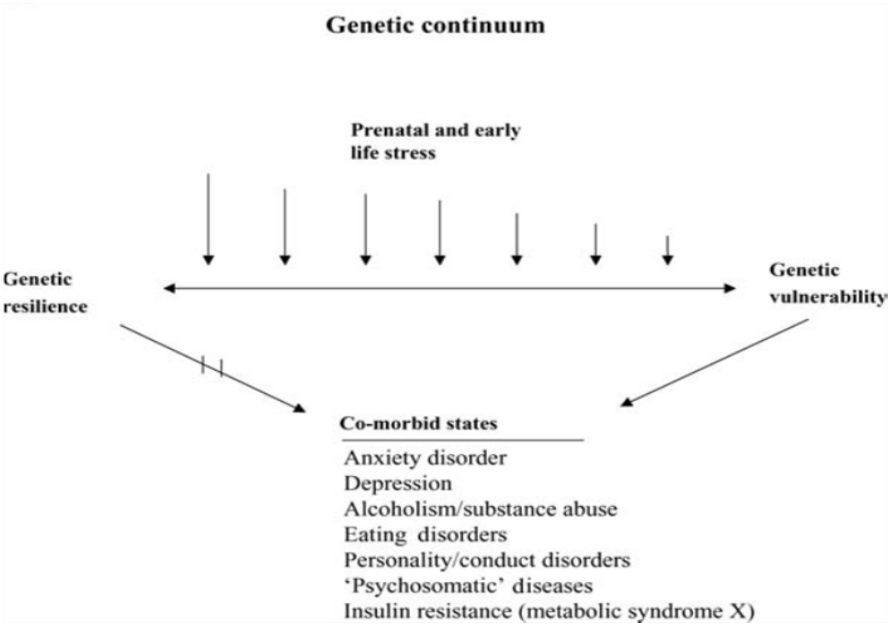
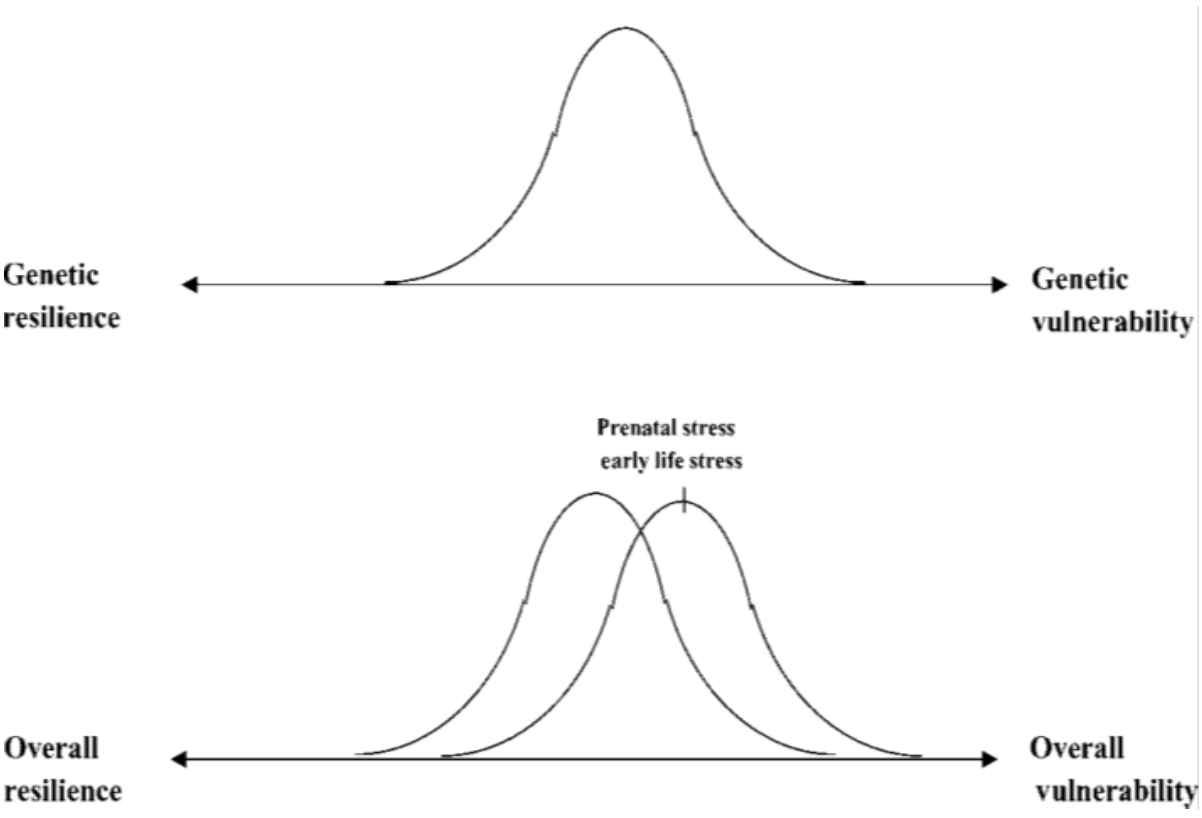
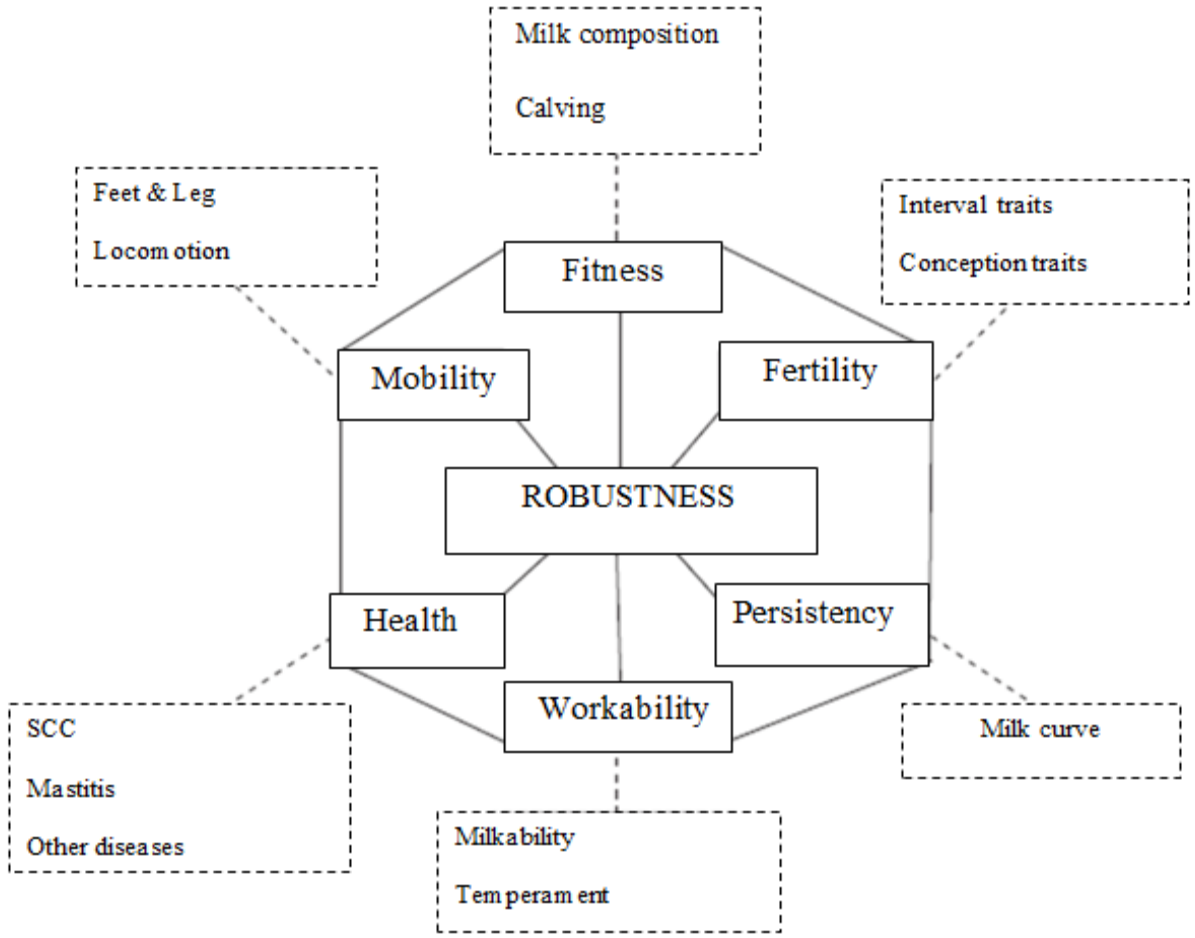


Figure 6. Environmental stressors in early stages of life have a permanent effect on the ability of the individual to cope with stress effectively, altering the vulnerability/resilience of an individual to stressors (Charmandari et al., 2005).



Another important consequence of animal response to stress can be the increase free radicals, which give rise to cellular damages and alteration of homeostasis (Sgorlon et al., 2007). However, the biological response to stress is variable and depends on the physiological conditions (not cognitive stress) and on how the animal perceives the environmental conditions (cognitive stress).

Figure 7. Traits related to robustness that are currently under investigation to be included in international breeding goals of cattle breeds (Calus et al., 2013).



A common feature of these situations is an increase of cortisol secretion. Although many methods for the evaluation of animal welfare are reported in the literature (Fraser et al.,1997; Von Keyserlingk et al., 2009; Gonzalez et al., 2008), as behavioral, housing and physiological approaches, cortisol is still one of the gold standard biomarkers to describe animal response to stress. Cortisol possesses powerful anti-inflammatory and immunosuppressive effects, by an inhibition of proinflammatory cytokines and tumor necrosis factor suppressive effects on thymus derived lymphocyte (T-cell) count and trafficking as well as the induction of thymocyte apoptosis. Proinflammatory cytokines are also know to stimulate GC secretion and the interactions between immune system and HPA axis have been strongly implicated in the pathogenesis of numerous stress related diseases, including inflammatory, autoimmune and allergic conditions.

5. Determination of cortisol in different matrices

Cortisol measurement has been extensively reviewed as a physiological measure of acute stress response in animals (Moberg, 2000). Despite its well-recognized role in stress in both animals and humans, the ability of cortisol to reflect stress levels over long periods of time is limited. This largely arises from the pulsatility of cortisol in the traditional matrices in which the hormone is sampled. Cortisol can be analyzed from many different media, most notably from blood, saliva, urine and feces.

Handling and restraint of dairy cattle has also been shown to rapidly increase blood cortisol, leading to confounding results (Cook et al., 2000). In addition, measuring cortisol in serum samples assesses total serum cortisol that includes both protein-bound and bioactive (free) cortisol. Consequently, total serum cortisol is affected by changes in levels of cortisol-binding globulin that can result in increases in total cortisol concentration measured, even though there is

no increase in stress or free cortisol concentrations. In addition, the sampling procedure *per se* could be a source of stress and increase cortisol.

Salivary cortisol concentrations correlate well with serum concentrations (Vining et al., 1983). In contrast to serum cortisol, salivary cortisol reflects free (unbound) cortisol and is collected by a less-invasive method. However, salivary cortisol concentrations still fluctuate significantly throughout the course of the day.

Both saliva and serum samples provide a measurement of the cortisol concentration at a single point in time. They can therefore be used to test acute changes, but are subject to daily fluctuations in relation to potential environmental challenges, making the assessment of overall long-term systemic cortisol exposure difficult. For urine, 24-h urine collections provide an integral of the free cortisol concentrations through the day, thus overcoming the issue of environmental stressors. Although fecal samples are less responsive to small changes in circulating cortisol and reflect cortisol concentrations from 10 to 12 h prior to fecal collection, they still cannot be practically used to measure the long-term changes that are characteristic of chronic stress (Möstl et al., 2002). However, repeated measures of cortisol for a defined window time in urine, feces and saliva can assist in depict a medium to long term response of stressors depending from the length of the collection time.

Figure 8. Consideration of the properties of existing matrices for cortisol measurement (adapted from Russel et al., 2011).

Property	Serum	Saliva	Urine	Feces	Milk	Hair
Level of invasiveness associated with sample collection	High	Low	Moderate	Low	Low	Low
Cortisol affected by stress procedure	Possibly	Possibly	Possibly	No	No	No
Storage	Spinning and refrigeration followed by freezing	Refrigeration or freezing	Refrigeration or freezing	Refrigeration or freezing	Refrigeration or freezing	Room temperature stable
Time periods of cortisol production represented	Single point measure	Single point measure	12-24 h;integral of exposure	12-24 h;integral of exposure	12-24 h;integral of exposure	Stable for years, Months/years
Affected by changes in cortisol binding globulin	Yes.Total Cortisol measured	No.Only free cortisol measured	No.Only free cortisol measured	No.Only free cortisol measured	No.Only free cortisol measured	No.Only free cortisol measured
Clinically relevant reference ranges established	Yes	Yes	Yes	No	No	No

For dairy cows, milk cortisol concentration is the most easily accessible and useful indicator for assessment of management system of the herd and of individual adaptability, because milking is a daily procedure. The limitation is that milk samples are restricted only to lactating animals.

It has been showed that measurements of cortisol concentration in milk and blood correlate closely (Verkerk et al.1998). If cows are milked for short intervals, milk cortisol instantaneously reflect changes in blood cortisol (Termeulen et al.,1981). Shutt and Fell (1985) found a 1:1 relation for absolute levels of free cortisol in blood and milk and total milk cortisol is about one-sixth of the respective blood values. For these reasons, milk cortisol can be used as a marker of acute stress, unless repeated samples in a defined period are measured.

The need for reliable methods to measure chronic stress in farm animals has contributed to the study for other noninvasive sampling sites, which are related to a long-term increases of cortisol (Moberg, 2000). One promising sampling site to assess cortisol levels are integuments and hair in particular. Koren et al. (2002) measured cortisol in hair and suggested that this matrix was particularly valuable for providing a marker that can represent "accumulations over time". Hair grows in cycles, with periods of new growth (anagen), transition (catagen) and quiescence (telogen). Cortisol is believed to enter the hair shaft in direct proportion to its free concentration in the blood (Figure 9), a process restricted to the phase of active hair growth in individual hair

follicles (Davenport et al., 2006; Pragst and Balikova, 2006). In cattle, body hair has shorter growth rest cycles than hair from the tail switch (Fisher et al., 1985). Moya et al. (2013) have observed that the hair sampled from the tail contained higher cortisol after the established time in comparison to hair sampled from the head or the shoulder. The authors suggested that tail is the most suitable location to collect hair samples to measure chronic stress. Furthermore, the cortisol concentration measured in the hair of the tail showed the best correlation with salivary and fecal cortisol (Moya et al., 2013). Determination of cortisol concentrations in the hair is thus a simple and noninvasive technique to assess changes over long term periods, without being subject to the interference of the momentary stress of capture during the sampling procedure.

Figure 9. Proposed mechanisms for incorporation of cortisol into hair via blood (A), sebum (B), and sweat (C) (from Russel et al., 2011).

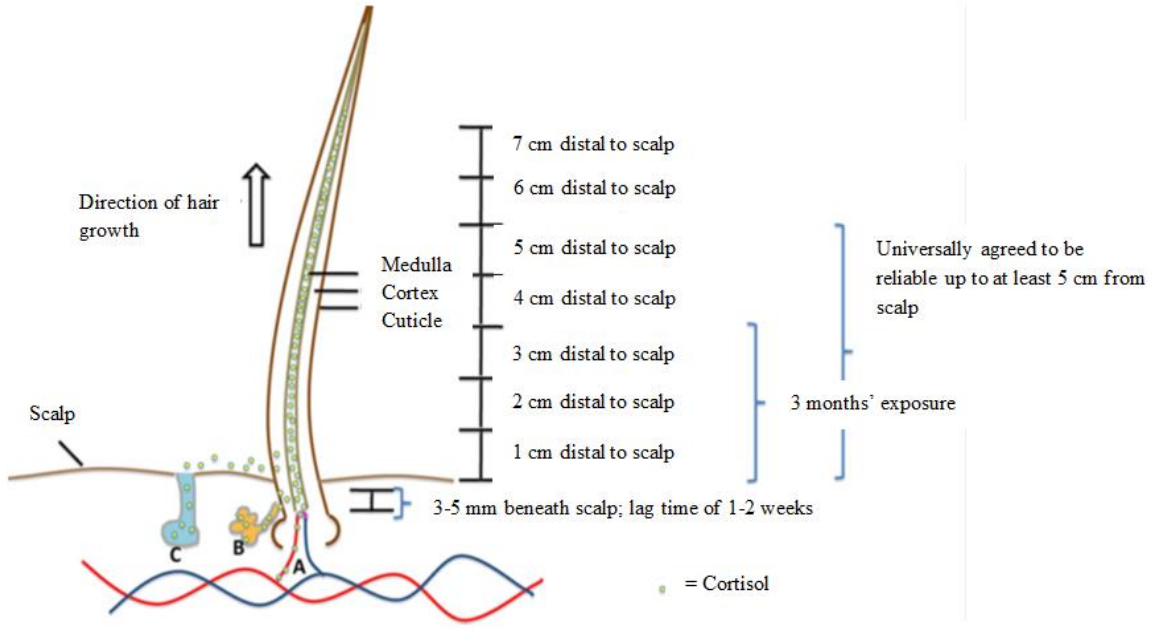
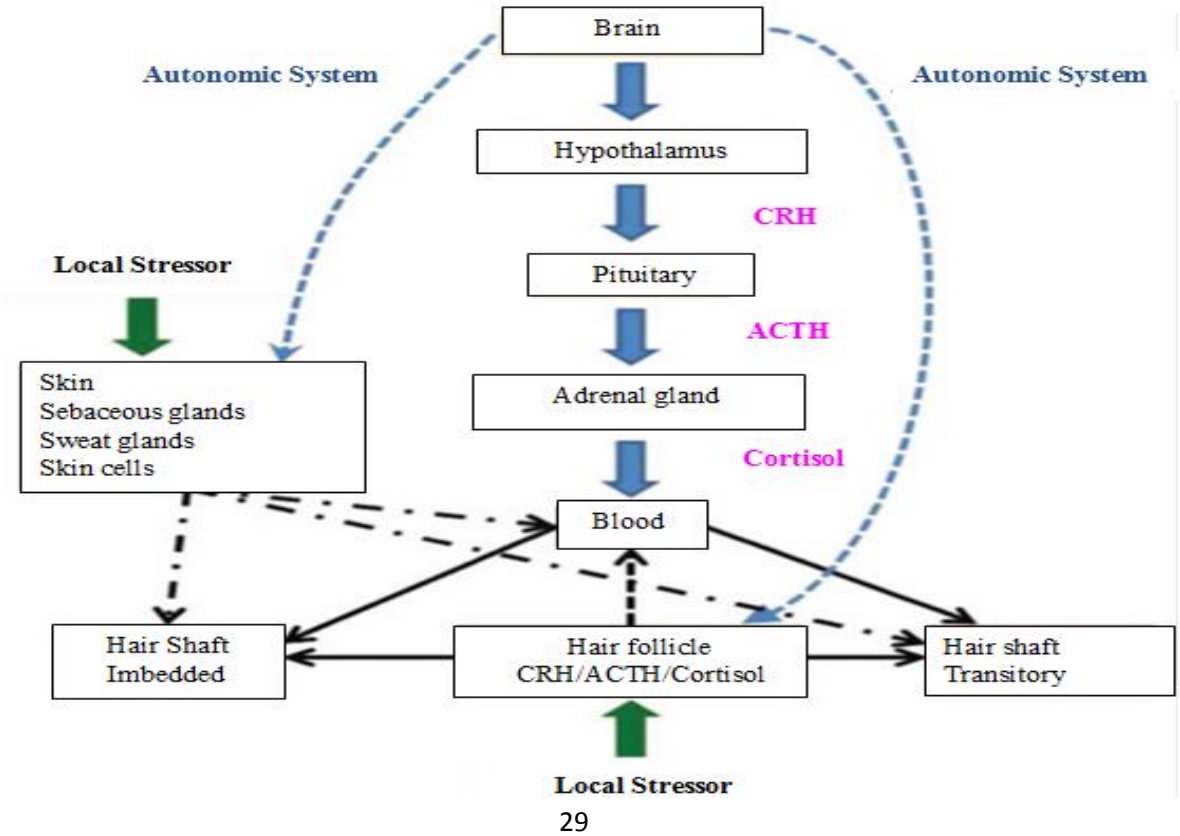


Figure 10. Diagram illustrating the possible pathways cortisol may be secreted into the hair shaft.



The hair shaft appears to have two repositories of cortisol. Firstly, one which is imbedded within the shaft and may reflect historical cortisol concentrations, and another which is transitory and reflects current cortisol concentrations. The source of the cortisol in either compartment remains unclear with possible sources being the skin, blood or the hair follicle. If the source is the skin or the hair follicle, it is also unknown whether the stimulus for cortisol production is central (via the sympathetic nervous system) or induced by the direct effects of a local stressor.

6. Rationale of the studies

In this PhD Thesis we investigated the relationships among factors affecting feed efficiency, metabolism and animals welfare, including the results of genetics selection in Holstein and Simmental dairy cows. These areas are very important not only from a scientific point of view but also for practical purposes, considering the impact that animal efficiency and welfare can have on the bioeconomy of the farming systems.

The research was split in two parts and two studies.

The aim of the studies in part 1 was to examine the effect of EBV for milk protein (EBVp) and breed on hematological, milk and urine metabolites in lactating cows. We hypothesized that animals of different breed and genetic merit for milk protein, as reflected by their EBVp, can impact markers of energy and nitrogen metabolism and of oxidative stress in biological fluids. Two studies were conducted in commercial farms of Italian Simmental and Italian Holstein cows after the peak of lactation, to avoid metabolic imbalance related to the onset of lactation.

In the preliminary study 1, the aim was to investigate the relationship - if any - between the application of breeding schemes to improve productive and functional traits in dairy cows and biomarkers of adaptive response of animals to environment. For this goal, milk, blood and urine were collected and analyzed for specific biomarkers of welfare and metabolic efficiency from a

relatively small number of lactating cows housed in commercial farms. The relationship of these biomarkers with the EBV for milk protein of the cows was thus investigated.

In study 2, a subset of biomarkers was used and their relationship with EBVs for milk protein, milk fat and somatic cell count was investigate in a larger number of dairy animals, still housed in commercial farms.

In the part 2, the relationship among the biomarkers of animal welfare and the somatic cells in milk was investigated, considering that the results obtained in the first part indicated a strong influence of environment for these variables. Also for part 2 the activity was split in two studies. Study 1 was more focused in the assessment of the relationship between milk and plasma cortisol, whilst in the study 2 a comparison between milk and hair cortisol was realized.

Data were obtained from the project Bellimpresa (Interreg Italia – Slovenia 2007-2013) and from the project PRIN-MIUR Gen2Phen.

Materials and methods

Animals

Study 1.

One hundred fifty three lactating cows were selected from 3 Simmental and 2 Holstein commercial dairy farms located in Italy, Friuli Venezia Giulia Region. Farms were sorted for having homogeneous management and diet compositions before and during the experimental period. The local Breeder Association (Associazione Allevatori del Friuli Venezia Giulia, Codroipo, Italy) provided assistance for farms selection and information about individual milk records through the lactation, reproductive parameters and managerial aspects. The Italian Holstein (IH) and Italian Simmental (IS) breeder associations provided updated EBV for milk protein content (EBVp) of the cows involved in the study. Lactating cows with days in milk (DIM) ranging from 70 to 250 days, clinically healthy and with parity from 2 to 6 (mean 3.0±1.1) were selected. Within each farm, cows were ranked according to the EBVp from minus to positive and selected every 5 EBVps from minus to positive values (about 20% lactating cows for each farm). The composition of the herds and the characteristics of the farms involved in the study are reported in Table 1. The day of sampling, the body condition score (BCS) of each cow was recorded by the same experienced observer on a scale from 1 (thin) to 5 (fat) with 0.25 point intervals (Edmonson *et al*., 1989).

All procedures were performed in respect of the Italian legislation on animal care (DL n 116, 27/1/1992).

Study 2

For study 2, 6 commercial farms of IS (F2, F3, F6, F7, F8 and F9) and 4 commercial farms of IH (F1, F4, F5 and F10), with a herd size ranging from 157 to 654 cows, for a total of 1041 cows

were selected. The inclusion criteria considered for the cows was clinical healthy and 50 < DIM < 270. Details of herd compositions and farm characteristics are reported in Table 2.

The Italian Holstein (IH) and Italian Simmental (IS) breeder associations provided updated EBV for milk fat content (EBVf), milk protein content (EBVp) and for SCC (EBVc) of the cows involved in the study.

All the lactating cows were housed in free stalls with cubicles and milking parlour and the management of the farms was similar. Cows had free access to water and a *ad libitum* total mixed ration (TMR) based on corn silage and formulated to cover nutrient requirements (INRA, 1989) was offered twice a day, after the morning and the afternoon milking. The composition of the rations and the amounts offered were recorded from the register of the TMR mixed feeder, starting from 1 week before the day of sampling. Samples of TMR were collected the day of sampling from the manger and were analyzed to calculate nutritive values, to ensure that energy and protein requirements were satisfied.

The day of sampling, the body condition score (BCS) of each cow was recorded by the same experienced observer on a scale from 1 (thin) to 5 (fat) with 0.25 point intervals (Edmonson *et al*., 1989).

Sample collections

The day of official milk recording made by the Breeder Association, 100 ml of milk samples were collected from each cow at the morning milking. An aliquot of 50 ml of milk was transferred into a tube containing preservative and was used for protein, fat, lactose analyses and for somatic cell count (SCC) determination. A second aliquot of 50 ml of milk was collected without preservative, frozen within 2 hours and stored at -20°C for cortisol analyses.

After milking and before the morning meal, when cows had *ad libitum* access to fresh water and spontaneously moved to cattle feed headlocks fence, blood was sampled from the coccygeal vein in 10 ml vacuum tubes with Li-heparin and K3-EDTA (Venoject, Terumo Europe N.V., Leuven,

Belgium). Blood was centrifuged within 1 h at 3000 RPM for 10 min at 20°C and plasma samples were stored at –20°C until further analyses.

Urines were sampled after stimulation of micturition. Ten ml of sample was immediately added with 10% sulphuric acid until a final pH of 3.0 was reached and the amount of acid added was recorded. The samples were filtered using a 0.22 μm membrane filter (Millipore Corporation, Billerica, MA, USA) and 3 aliquots of each sample were stored at -20°C until analysis.

The day of sampling, the body condition score (BCS) of each cow was recorded by the same experienced observer on a scale from 1 (thin) to 5 (fat) with 0.25 point intervals (Edmonson et al., 1989).

All procedures were carried out in respect of Italian legislation on animal care (DL n.116, 27/1/1992).

Analysis of samples

Samples of TMR were analyzed for dry matter (DM; 105°C for 12 h), ash (512°C for 8 h), CP, ether extract (EE), NDF and starch with standard methods (AOAC, 2012). The net energy of lactation and the digestible protein in the intestine (PDIE, PDIN) were calculated from the chemical data and from data in tables using the INRA (1989). The ingredients and the composition of the diets are reported in Tables 3 and 4 for Study 1 and 2, respectively.

Milk protein, fat, lactose contents and SCC of milk samples were analyzed with mid infrared spectroscopy (MIR, Fourier Transform Instrument, FT6000, Foss Electric, Hillerød, Denmark).

Total protein, albumin, urea, glucose, creatinine, cholesterol, AST, gGT, in plasma were analysed using a Roche Cobas® 6000 analyzer with proprietary kits (TP2, ALB2, GLUC3, UREAL and CREP2; F. Hoffmann-La Roche AG, Basel Switzerland) and ceruloplasmin, haptoglobine and paraoxonase according to the method reported by Bionaz et al. (2007).

NEFA, BOHB, glutathione peroxidase (GPx) and total antioxidant status (TAS) in plasma were measured with Randox kits (FA 115, RB1008, RS504, NX2332; Randox Laboratories Limited,

Crumlin, UK). GPx was expressed as units of Hb. Plasma Zn was analysed with the Sentinel kit (17640H, Sentinel CH. SpA, Milan, Italy).

Urine samples were analysed for total N with Kjeldahl method, creatinine with Jaffe method (Hawk *et al.*, 1976) and urea with Berthelot method (Randox kit UR 1068, Randox Laboratories Limited, Crumlin, UK). Uric acid and allantoin were measured using the HPLC method (Piani *et al.*, 2004). Purine derivative (PD) N was calculated as the sum of allantoin N and uric acid N.

Cortisol assay

Skimmed milk was previously obtained by centrifugation (1,500 g, 4°C, 15 min). Cortisol was extracted two times from skim milk (0.2 mL) with 4.0 mL dichloromethane in a glass tube. The mixture was shaken at 250 • g for 15 min in a shaker, and the supernatant solution was transferred into a fresh glass tube. The extracted solution was evaporated by heating in a hot water bath (50°C) for 2 h. After complete drying, 0.1 mL assay buffer (PBS, 0.1% BSA, pH 7.4), 0.1 mL borate buffer (boric acid 1.55 g in 500 mL distilled H_2O, 0.1% BSA) added with 0.01 g thimerosol (sodium ethylmercurithiosalicylate; Sigma-Aldrich) were put into the tube and mixed by shaker for 10 min (Waki et al., 1987).

Plasma samples (0.1 mL) were extracted with 8 mL diethyl ether. The ether fractions were transferred into fresh glass tubes and dried under nitrogen. The dry extracts were carefully dissolved in 0.2 ml assay buffer (Gabai et al., 2004).

Skim milk (0.05 mL) and plasma (0.1 mL) extracts were assayed by a solid-phase microtitre RIA (Gabai et al., 2006). Briefly, a 96-well microtitre plate (Optiplate, Perkin-Elmer Life Science, Boston, MA, USA) was coated with anti-rabbit γ-globulin serum raised in a goat, by incubating overnight the antiserum diluted 1:1000 in 0.15 mM sodium acetate buffer, pH 9, at 4°C. The plate was then washed twice with PBS 0.1% BSA, pH 7.4 (RIA buffer) and incubated overnight at 4°C with 0.2 mL of the anti-cortisol serum diluted 1:8000. The antiserum (Centro Medico Diagnostico Emilia, Bologna, Italy) was raised in the rabbit against cortisol-3

carboxymethyloxime–BSA and showed the following cross reactions: cortisol 100%, prednisolone 44.3%, 11-deoxycortisol 13.9%, cortisone 4.9%, corticosterone 3.5%, progesterone <0.01%.

For the extraction of cortisol the following procedure was used (Accorsi et al.2008):

- Trim the hair with a blade;
- Weight 100 mg of trimmed hair into a 15 ml glass vial;
- Add 5 ml methanol and incubate in water bath at 45°C for 18-24h;
- Transfer the vial content into a 10 ml tube and centrifuge 15 min at 3000 rpm;
- Transfer the supernatant to 2 ml microcentrifuge tube and dried with a vacuum concentrator (Centrifugal System Jouan RC 10.10);
- Reconstitute the dried sample with 0.6 ml of phosphate buffer with 0.1 % BSA;
- Freeze the samples at -20°C until analysis.

Plasma, milk and hair extracted samples were transfer to a 396 wheel microplate. The plate was carefully washed with RIA buffer, and standards (1.56–400 pg/well), quality control, unknown extracts and tracer (1,2,6,7–3H-cortisol, Perkin-Elmer Life Sciences, 30 pg/well, specific activity: 3700 GBq/mmol) were added (final volume: 0.200 mL). The plate was incubated overnight at 4°C, the incubation mixture was decanted and wells washed with RIA buffer, added with 200 µl scintillation cocktail (Microscint 20, Perkin-Elmer Life Sciences) and counted on the beta-counter (Top-Count, Perkin-Elmer Life Sciences). All samples were assayed in duplicate. The sensitivity of the assay was defined as the dose of hormone at 90% binding (B/B0) and was 3.125 pg/well. The intra-assay and inter-assay coefficients of variation in high and low cortisol pooled plasma samples were 5.9% and 9.1 % and 13.5% and 15.1%, respectively.

Table 1. Composition of the herds and characteristics of the farms involved in the study 1

		Farm				
		FA	FB	FC	FD	FE
Breed		IS	IS	IS	IH	IH
Herd size	No	343	270	216	368	433
Dairy animals	No	183	169	119	194	215
Heifers	No	65	61	43	76	97
Lactating cows	No	152	148	99	155	182
Cows < 70 DIM	No	31	16	12	14	22
Cows sampled	No	27	33	20	36	39
	%	22.3	25.6	23.0	25.5	24.4
DIM	Mean	126.7	141.4	141.8	151.0	145.8
EBVp	Mean	17.9	8.6	-1.2	22.6	16.5
Housing	type	Free stall	Free stall	Free stall	Free stall	Free stall
Bedding	type	Concrete	Straw	Concrete	Concrete	Concrete
Milking	type	Parlour	Parlour	Parlour	Parlour	Parlour

DIM: Days In Milk; EBVp: Estimated Breeding Values for protein

IH: Italian Holstein; IS: Italian Simmental

Table 2. Composition of the herds and characteristics of 10 farms involved in the study 2

		FARMS									
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Breed		IH	IS	IS	IH	IH	IS	IS	IS	IS	IH
Herd size	No	654	270	341	390	442	320	538	157	201	456
Dairy animals	No	347	147	194	236	235	163	280	88	119	250
First calving	No	131	36	64	74	82	51	86	18	41	85
Lactating cows	No	313	123	153	208	195	137	225	74	96	227
Cows>50 DIM	No	279	111	144	186	147	117	185	64	84	204
Cows sampled	No	184	88	95	128	75	92	126	63	78	112
DIM	mean±sd	(179±62)	(166±66)	(168±66)	(177±54)	(111±63)	(132±6)	(160±63)	(153±102)	(162±99)	(179±52)
Housing	Type	Free stall	Free stall	Free stall	Free stall	Free stall	Free stall	Free stall	Free stall	Free stall	Free stall
Bedding	Type	Concrete	Straw	Straw	Concrete	Concrete	Concrete	Concrete	Concrete	Concrete	Concrete
Milking	Type	Parlour	Parlour	Parlour	Parlour	Parlour	Parlour	Parlour	Parlour	Parlour	Parlour

IH: Italian Holstein; IS: Italian Simmental

DIM: Days In Milk

Table 3. Composition of the rations offered to the dairy cows and chemical and nutritive contents involved in the study 1.

	Farm				
	FA	FB	FC	FD	FE
Ingredients, kg DM/d					
Lucerne, hay	3.06	4.45	3.13	2.50	4.03
Grass, hay					0.90
Corn, silage	6.82	6.06	6.00	7.82	6.15
Corn cob, silage	3.13	3.24	3.54		
Lucerne, silage	1.50		3.00	3.16	
Grass, silage		0.71			
Corn, ground	0.87	1.04	0.88	3.15	4.56
Soybean meal s.e.	1.05	0.70	2.19		1.75
Rapeseed meal s.e.					0.90
Whole Soybean	1.25				
Barley, ground		0.44			
Bran wheat			0.88		
Protein and fat supplements	2.38	2.64	0.45	3.17	2.45
Minerals	0.20	0.05	0.10	0.55	0.05
Total	20.3	19.3	20.2	20.3	20.8
Composition, %/DM					
CP	15.6	15.1	15.7	14.7	15.4
EE	4.0	2.4	3.0	3.3	4.2
Ash	7.6	6.3	5.9	6.8	5.4
NDF	31.9	33.9	34.4	34.3	32.5
Starch	26.6	27.9	25.3	25.8	27.9
PDI, g/d					
PDIN	2077	1963	2109	1604	1954
PDIE	2024	1804	1921	1526	1784
NEl, MJ/d	125.7	114.6	119.2	114.1	120.2

CP = crude protein; EE = Ether Extract; NDF = Neutral Detergent Fiber, PDI = protein digested in the small intestine; PDIE = protein digested in the small intestine supplied by rumen undegraded dietary protein and by microbial protein from rumen-fermented organic matter (INRA, 1989); PDIN = protein digested in the small intestine supplied by rumen undegraded dietary protein and by microbial protein from rumen degraded N (INRA, 1989); NEl = Net Energy for lactation.

Table 4. Ingredients, chemical composition and nutritive contents of the rations offered to the dairy cows involved in study 2.

Item	Unit	Farm									
		F1	F2	F3	F4	F5	F6	F7	F8	F9	F10
Lucerne hay	kg/d	8.0	4.5	2.5	2.1	5.0	4.0	4.5	8.0	3.5	2.0
Lucerne silage											2.4
Pasture hay				2.0		1.5		1.5		0.8	
Pasture Silage				3.0						2.5	
Silage wheat											2.2
Grass,hay											
Corn,silage		22.5	20.0	25.0	15.0	15.0	21.0	20.0	20.0	32.0	20.0
Corn cob,silage											
Lucerne,silage					4.0						
Wheat x Ryegrass, silage					10.0						8.0
Sorgum,silage									5.0		
Triticale x Pea,silage							6.0				
Ryegrass,silage			5.0								
Grass,silage											
Cotton Seeds						1.0		1.0			1.2
Corn mash		3.2	4.2	4.0	4.0		6.0				
Corn meal		2.0	1.8		3.5			4.5			6.0
Soybean meal s.e		2.0	1.0	2.0	1.0	2.0	0.8	1.0			2.1
Rapeseed meal s.e					1.2		1.0	1.4			1.5
Guar meal					1.3						
Whole soybean							1.5				
Corn ground						6.0	1.0				
Barley ground							1.0				
Linseed						0.3					
Straw		0.5			0.5						
Bran wheat								1.5			
Nucleo		2.0	2.5	2.0					6.0	8.0	
Hydrogenated fats		0.3				0.1		0.1			
Molasses		1.0								1.5	
Urea		0.05					0.07				
Minerals				0.3		0.5	0.5	0.4			0.2
Total		41.5	39.0	40.7	42.6	31.4	42.8	35.9	39.0	48.3	45.6
DMI	kg/d	23.3	19.8	19.8	20.8	19.5	21.8	20.7	20.4	23.4	22.6
Starch	% / DM	25.2	30.1	26.6	28.1	28.6	28.9	26.8	20.8	25.5	27.8
CP	“	14.7	13.2	14.3	12.4	14.8	13.3	13.2	14.1	13.1	14.0
EE	“	2.9	3.0	3.0	2.9	4.1	4.1	4.1	2.7	2.8	3.9
Ceneri	“	6.6	6.4	7.5	6.4	7.5	7.7	7.4	8.1	7.8	6.7
NDF	“	38.7	38.8	41.3	40.0	35.4	38.1	36.9	42.6	38.5	37.4
UFL	Unit	20.0	17.5	17.3	17.2	17.9	18.3	17.9	16.9	21.3	20.7
PDIN	g/d	2222	1726	1850	1549	1972	1754	1766	1906	1996	2058
PDIE	g/d	2154	1785	1817	1658	1940	1720	1784	1785	1960	2043

DMI = Dry matter intake; CP = crude protein; EE = Ether Extract; NDF = Neutral Detergent Fiber; UFL= Feed unit for lactation ; PDIE = protein digested in the small intestine supplied by rumen undegraded dietary protein and by microbial protein from rumen-fermented organic matter (INRA, 1989); PDIN = protein digested in the small intestine supplied by rumen undegraded dietary protein and by microbial protein from rumen degraded N (INRA, 1989).

Statistical analysis

All the data were stored in a spreadsheet using Microsoft Office Excel (2010), Microsoft Corp., Redmond, WA) and statistical analyses were performed with the SPSS package (1997). Normality of data was tested by the Kolmogorov-Smirnov non parametric test. Only SCC and cortisol in milk, blood and hair were not normal distributed and a log(2) transformation was used before statistical analysis.

For the part 1 of the present thesis, the following models were applied.

Study 1. The general linear model was used for the outcomes of milk yield and its composition, BCS, serum, plasma and urine metabolites, with a fixed effect for breeds and farms nested within breed. Within breed linear regressions were used for DIM and for EBVp. All tests were 2-tailed and significance was based on a $P < 0.05$.

$$y_{ij} = \mu + B_i + F_j(_i) + D(_i) + V(_i) + \varepsilon_{ij}$$

Where:

μ = general meal

B_i = fixed effect of breed (1,2)

F_j = fixed effect of farm (1,5), nested within breed

D = linear effect of DIM, nested within breed

V = linear effect of EBVp, nested within breed

ε_{ij} = residual error

Study 2. Before statistical analysis, animals were classified for parity with ordinal value of 1 for first calving, 2 for second calving and 3 for cows with more than 2 calving. Analysis was performed with a mixed model, with fixed effects of Breed and Parity and random effect of Farm; the model also included a linear relationship with DIM and with EBVs within breeds. The EBVs considered were kg of protein (EBVp), kg of fat (EBVf) and somatic cell count index (EBVc).

$$y_{ijk} = \mu + B_i + Lj + F_k(_i) + D(_i) + P(_i) + G(_i) + S(_i) + \varepsilon_{ijk}$$

Where:

μ = general meal

B_i = fixed effect of breed (1,2)

L_j = fixed effect of parity (1,3)

F_k = random effect of farm (1,10), nested within breed

D = linear effect of DIM, nested within breed

P = linear effect of EBVp, nested within breed

G = linear effect of EBVf, nested within breed

S = linear effect of EBVc, nested within breed

ε_{ij} = residual error

For the part 2, cows were classified in 3 groups according to the somatic cell counts. Class 1 grouped the cows with a value of SCC lower than or equal to 200,000 cells/ml of milk (healthy), Class 3, grouped the cows with a value of SCC higher than or equal to 400,000 cells/ml of milk and Class 2 grouped the cows with intermediate value of SCC (200,000<cell/ml of milk<400,000). Cortisol concentrations in plasma for trial 1, in milk for trials 1 and 2 and in hair

for trail 2 were analyzed with a general linear, with the fixed effects for Class of SCC (from 1 to 3) and Breed (from 1 to 2), the random factor of Farm (from 1 to 4 in Study 1 and from 1 to 10 in Study 2). In the model, the linear effect for days in milking (DIM) and milk yield (MY) was also considered. Least square difference test was applied to assess significant differences between means.

$$y_{ijk} = \mu + B_i + Cj + F_k(_i) + D + M + \varepsilon_{ijk}$$

Where:

μ = general meal

B_i = fixed effect of breed (1,2)

C_j = fixed effect of class of SSC (1,3)

F_k = random effect of farm (1,4; 1,10), nested within breed

D = linear effect of DIM

M = linear effect of M

ε_{ij} = residual error

Results and Discussion

Part 1.

Relationship of breed and estimated breeding values with biomarkers of metabolism and inflammation in Italian Holstein and Italian Simmental cows after the peak of lactation

Results

In the first study, 155 cows were sampled from 4 farms (27, 33, 20, 36 and 39 for farm FA, FB, FC, FD and FE, respectively), corresponding to 34-46% of animals within 70 < DIM < 250 (Table 1). Cows were housed in a freestall barn with cubicles for FA, FC, FD and FE and straw for FB. The ingredients of the rations for the farms were corn silage, ground corn, alfalfa hay, solvent extracted soybean meal and protein supplements, based on this latter feed. The chemical compositions of the diets did not vary largely between farms. The CP content ranged from 14.7 to 15.7% of DM, the NDF from 31.9 to 34.4% and the starch from 25.3 to 27.9% on DM basis.

In the second study, the cows sampled from ranged from 75 to 184 for IH and from 63 to 126 for IS, corresponding to 60.2 and 80.6 % of the lactating cows within 50 < DIM < 270 for IH and IS, respectively (Table 2). The differences in the percentage were due to the shorter lactation length of the IS cows compared to IH cows that averaged 300 and 345 days, respectively. The cows of study 2 were also housed in a freestall barn, with straw for F2 and F3 and cubicles for the other barns. The ingredients of the rations for the 10 farms were similar to that of the farms of study 1, including corn silage, ground corn, alfalfa hay, solvent extracted soybean meal and protein supplements, based on this latter feed. The chemical compositions of the diets varied, on DM basis, from 12.4 to 14.8% of DM for CP, from 35.4 to 42.6% for NDF and from 20.8 to 30.1% for starch.

The mean DIM values of the sampled cows in study 1 varied from 126.7 days of FA to 151.0 days of FD, corresponding to the mid lactation phase. For the second study, the mean DIM values of cows sampled were lower than that of study 1 and ranged from 110.6 to 178.8, because the inclusion criteria considered all the healthy cows with 50 > DIM > 270 and also the first calving cows.

The effects of breed, farm within breed and the covariates for DIM and EBVp within breed are reported in Table 5 for BCS, milk yield and its composition. For the IH cows, significantly higher milk ($P < 0.001$) and fat ($P < 0.01$) yields and significantly lower values ($P < 0.001$) of BCS, protein percentage, SCC and urea were observed in comparison to IS. The linear effect of DIM was positive for BCS ($P < 0.05$), milk protein percentage and SCC ($P < 0.01$) and negative for milk fat and protein yields ($P < 0.05$). A positive linear effect of EBVp was observed for milk and protein yields ($P < 0.01$), fat yield ($P < 0.05$) and milk protein percentage ($P < 0.01$) for IH cows. For the IS cows, the EBVp was inversely related with BCS ($P < 0.05$) and was positively related to milk yield ($P < 0.01$), milk fat yield ($P < 0.05$) and protein yield ($P < 0.01$).

The effects of breed, farm within breed and the covariates for DIM and EBVp within breed for haematological variables are reported in Table 6. Higher mean values for creatinine ($P < 0.001$) and lower mean values for Zn, TAS and GPx ($P<0.001$) were observed in IS in comparison to IH cows. The DIM was linearly related only to plasma urea ($P < 0.05$). The EBVp was negatively related to glucose in the IS cows ($P < 0.05$) and to BOHB in both breeds ($P < 0.05$). The concentrations of N, creatinine and the creatinine to N ratio in urine samples (Table 7) were significantly higher ($P < 0.01$) in IS than IH cows. The PD N to total N ratio ($P < 0.05$) and PD N to creatinine N ratio ($P < 0.001$) were lower in IS than in IH cows. A linear effect of DIM was calculated for the creatinine to total N ($P < 0.05$) and EBVp was negatively related to urea to total N ratio and PD N to total N ratio ($P < 0.05$) in IS cows and to PD N to creatinine N ratio ($P < 0.05$) in IH cows.

The effects of breed, farm and parity and of the covariates for DIM and EBVf, EBVp, EBVc within breed on BCS, milk yield and its composition are reported in Table 8. The IH cows showed numerically higher milk yield, fat and protein yields and milk urea and lower BCS, fat and protein percentages, casein percentage and SCC, in comparison to IS. The observed differences, although in some case numerically relevant, were never significant.

The linear effect of DIM was significant for BCS, milk, protein and fat yields and for the percentage of protein and fat. In particular, a positive effect of EBVf was observed for milk yield, fat percentage and yield ($P < 0.001$) for both breeds, and for protein percentage for IH and for protein yield for IS ($P < 0.01$). A positive linear effect of EBVp was observed also for milk yield and protein yield in two breeds. EBVp was inversely related with fat percentage in IH and IS ($P < 0.001$). The SCC was significantly related to EBVc ($P < 0.001$), although positively in IH and negatively in IS. Furthermore, EBVc was positively related to milk urea in IH and to milk yield in IS ($P < 0.05$) and negatively related to protein percentage in IH ($P < 0.05$).

The effects of breed, farm and parity and the covariates for DIM and EBVf, EBVp and EBVc, within breed on plasma and blood parameters are reported in Table 9.

Higher mean values for BOHB, creatinine ($P < 0.01$), bilirubine, glucose and lower mean values for cholesterol ($P<0.05$), urea, AST/GOT, gGT and NEFA were observed in IS in comparison to IH cows. A significant effect of parity was observed for cholesterol, bilirubine ($P < 0.001$) and gGT ($P < 0.01$).

The DIM was linearly related to NEFA and to bilirubine ($P < 0.001$) and to plasma urea, gGT, BOHB and creatinine ($P < 0.001$). EBVf was positively correlated ($P < 0.001$) with BOHB and negatively related to glucose in both breeds. EBVf was negatively related also to cholesterol and AST/GOT in IH cows ($P < 0.05$) and to creatinine and glucose in IS cows ($P < 0.05$). A positive linear effect of EBVp was observed for gGT ($P < 0.001$), AST/GOT and cholesterol ($P < 0.01$) in IH cows; instead a negative linear effect of EBVp was observed for urea in plasma and BOHB in both breeds. EBVc was positively related ($P < 0.05$) only with plasma urea in IH cows.

The effects of breed, farm and parity and the covariates for DIM and EBVf, EBVp, EBVc, within breed on protein, zinc, acute phase proteins, paraoxonase and milk and hair cortisol are reported in Table 10.

Numerically higher means value of albumine, paraoxonase and cortisol in milk were observed in IS cows in comparison to IH cows. The effect of the parity was significant for all the parameters. The linear effect of DIM was highly significant ($P < 0.001$) for paraoxonase, Zinc and cortisol in hair ($P < 0.05$). A positive linear effect of EBVf was observed for total protein in IS ($P < 0.05$) and a negative effect was observed for paraoxonase in IH cows ($P < 0.001$) and haptoglobine in IS cows ($P < 0.05$). A positive linear effect of EBVp was observed for paraoxonase in IH cows ($P < 0.001$) and for cortisol in milk in IS cows (($P < 0.05$). A positive linear effect of EBVc was observed only for globuline ($P < 0.01$), albumine and total protein ($P < 0.05$) in IS cows.

Discussion

This study investigated whether differences in genetic merit and breed of mid lactating cows are related to variations of metabolites in milk, blood and urine. A relationship between metabolic profiles in biological fluid (blood, urine, milk) and productive efficiency of the animals has been extensively reported. According to Spicer et al. (1990) and Herd and Arthur (2009), the significant variations of blood metabolites are not only related to feed intake, diet composition, and physical activity, rather depend at a certain degree from the genotype of the animals.

Among the EBVs, we concentrated our attention in the first part to the milk protein yield, as this trait is the combination of protein percentage and milk yield. A high positive genetic correlation between milk and protein yield has been reported (Lipkin *et al.*, 2008), while for milk fat and protein percentage the correlation with milk production is negative (Viitala *et al.*, 2003). At the same time the lack of QTL affecting only protein yield was confirmed by both these studies and Lipkin *et al.* (2008), whom in Israeli Holstein cows reported that 68.9 and 76.5% of QTL markers affecting protein yield were also associated to protein percentage and milk yield,

respectively. The IS is a dual purpose breed and the selection combines milk and meat production traits (www.anapri.it) with a breeding scheme differing from that of IH breed, which does not consider meat traits (www.anafi.it) and for this reason a linear regression of EBVp within breed was used in the statistical model.

The biomarkers measured in the present study are often used for diagnostic purposes or to verify the health conditions or the response of animals to specific treatments. Furthermore, biomarkers are often measured during the peripartum, when cow's response is largely affected by environmental conditions, as BCS, diet composition and feeding regimes, calving, management system and milking hygiene (Stefanon *et al.*, 2005). Few information is reported about the effect of cow genetic background on biomarkers of metabolism measured in blood, urine and milk in mid lactating cows. Our hypothesis is that environmental effects are minimized after the transition period, when cows enter into a more stabilized phase of lactation. This offers the opportunity to investigate the relationship of genetic merit with biomarkers of stress response and energy and protein metabolism.

Productive performance at the time of sampling were related to EBVp and breed (Table 5), confirming that the selection criteria of the cows, based on estimated performance, reflects the differences of their genetic background. The significantly higher SCC in IS in comparison to IH cows could be related to the different breeding scheme applied in Italy, since the weight of SCC trait accounts for 5% in IS and 10% in IH (www.anapri.it; www.anafi.it). Another explanation could be the different ability to cope with milk yield for the two breeds. In a study of Olmos *et al.* (2009), dairy Holstein cows from herds with lower genetic merit had the highest serum amyloid A concentration and a tendency for higher SCC in early lactation, suggesting a worse udder health. The significant difference of plasma Zn between breeds (Table 6) supports a more favorable immune surveillance in IH cows. Administration of dietary Zn has been reported to reduce milk SCC (Sobhanirad *et al.*, 2010), since this mineral improves immune function by activating cell-mediated immune responsiveness and plays a role in keratin formation of the teat

canal. The blood GPx, an antioxidant enzyme inversely related to oxidative stress in dairy cows (Stefanon *et al.*, 2005) and TAS, which expresses the total antioxidant capacity of plasma, would also support a lower involvement of inflammatory cascade in IH cows. It is well known that an important component of the immune response is oxidative burst, during which superoxide anion radicals are produced from oxygen, and consequently cause a perturbation in the oxidative balance of the animal.

The BOHB and NEFA contents in plasma are reliable markers of energy metabolism at the beginning of lactation (McArt *et al.*, 2013), when a large mobilization of fat stored in the tissues in high yielding cows occurs, but less information is reported in the later phase of lactation, when the recovery of DMI allows to cover the energy requirements for maintenance and for milk production. A significant and negative linear relation was found between BOHB in plasma and EBVp for IS and IH cows (Table 6), and it is likely that higher genetic merit can use energy more efficiently. BOHB concentration in plasma is mainly used to diagnose sub clinical ketosis during the peripartum (Duffield, 2000), but evidences in growing cattle suggest that it can also be considered a marker of metabolic efficiency and residual feed intake (Kelly *et al.*, 2010). Moreover, BOHB can reduce feed intake and depress pituitary and thyroid functions, which are both strongly implicated in homeostatic control (Laeger *et al.*, 2010).

Complete nitrogen balance technique is used to assess nitrogen efficiency for lactation, but total urine collection is not feasible in trials involving cows in commercial farms. Alternatively, the concentration of N and of nitrogenous compounds can be used as an indicator of whole body and rumen nitrogen metabolism and usage (Gruber *et al.*, 1999). The significantly higher urinary total N content in IS in comparison to IH cows is related to the higher creatinine N concentration of the dual purposes cows (Table 7). Moreover, the significantly higher urine concentrations of creatinine in IS cows corresponds to a higher plasma creatinine concentration and is probably related to the body composition of the dual purpose breed in comparison to IH. As a product of muscle metabolism, creatinine excretion has been directly related to muscle mass, as diet

composition has a relatively minor effect on creatinine excretion (Chen *et al.*, 1995). As reported from the IS breeder association (www.anapri.it), the muscularity accounts for 6% in the selection scheme whilst is not considered for IH cows.

Urinary excretion of PD N has been proposed as a marker of rumen microbial protein supply (Stefanon *et al.*, 2001), but also this technique requires total daily urine collection. Alternatively, PD N to creatinine N ratio can be used in a spot sample (Chen *et al*., 1995). The significantly higher PD N to creatinine N ratio measured in urine samples of IH cows support a higher microbial protein supply for this breed. Furthermore, the negative effect of EBVp observed for urea N to total N ratio and for PD N to total N ratio in IS and for PD N to creatinine N ratio in IH could indicate a more efficient nitrogen utilization in the cows with higher genetic merit.

In the second study, a larger number of cows and farms were involved and the EBV for fat yield (EBVf) and SCC (EBVc), other than EBV for protein yield (EBVp), were considered in the statistical model. It must be underlined that only one sample was collected for each cows and even though not repeated measurements were performed, the results of table 8 confirmed the strong relationship of genetic values with productive traits for both the breeds, also observed in the study 1 (Table 5). However, in the second study breed and farm effects were not significant, apart for milk urea, probably as a consequence of the high and significant linear relationship of the independent variables with DIM and EBVs. In the second dataset, the inclusion criteria for the cows were slightly different, since also first calving cows were sampled and the DIM was 50 < DIM < 270, in comparison to that of the study 1 (70 < DIM < 250). Also for the variables measured in plasma, the effect of breed and farm was limited (Table 9), but the creatinine concentration in IS confirmed the results of study 1.

Interestingly, the negative linear relationship between BOHB in plasma and EBVp for IS and IH cows found in study 1 was confirmed ($P<0.05$). In the second study, the positive linear relationship with EBVf in both breeds would indicate that fat and protein synthesis in milk are inversely related or, at least, compete for the same energy substrate. Under conditions of high

energy demands, as during prolonged exercise, the perinatal period, or lactation, energy stores need to be mobilized from fat and muscle depots, and ketone bodies become an important respiratory fuel (Hawkins & Biebuyck, 1979). BOHB is produced in liver when fatty acid load overcomes the oxidative ability and it is rapidly exported to peripheral tissues where can be used as energy substrate, especially if a shortage of glucose occurs. There are two main BOHB forming metabolic pathways. First, BOHB can be generated by reduction of acetoacetate, the first product of ketogenesis in the liver of all species, and second, by oxidation of butyrate in ruminal epithelial cells exclusively (Van Soest, 1994). Therefore, the plasma concentration of BOHB (0.5–2 mM) in cows increases during and after food intake and is affected by dietary composition and physiological stage (Duske et al., 2009). When metabolic carbohydrate utilization is perturbed, as in diabetes mellitus, ketone body concentration may increase to excessive levels associated with limited metabolic degradation leading to a pathological syndrome known as ketosis (Van Soest, 1994). In the bovine clinical ketosis is defined at 2 mM BOHB serum concentration (Dirksen, 2006). Endogenous production of BOHB from hepatic fatty acid oxidation is inhibited by feed-back regulation of lipolysis via BOHB and the adipocyte G-protein-coupled receptor 109A (GPR109A) (Taggart et al., 2005). Within this negative feedback mechanism, BOHB acts to diminish the concentration of plasma free fatty acids and prevents an excessive exhaustion of fat depots during prolonged starvation. The BOHB receptor GPR109A is also known as a niacin receptor. Niacin is also used in ketotic cows to lower blood BOHB and to elevate glucose concentration without affecting food intake (Dufva, Bartley, Dayton, & Riddell, 1983). There are few reports in the literature on the relationship between RFI phenotype and systemic concentrations of BOHB, despite the fact that it is used more preferentially as an energy substrate by muscle tissue in ruminants compared with monogastrics.

A positive linear effect of EBVp in IH cows to the AST/GOT, gGT and cholesterol suggests that the selection of this breed had leaded to a more pronounced liver activity, probably to face with

the higher metabolism to sustain milk production (Medonca et al., 2013). For the other variables (Table 10), the relationship with EBVs were more limited.

Protection from oxidative stress in mammalian cells is due to a wide range of defense mechanisms, which include the activities of antioxidant enzymes. Paroxonase is a mammalian high-density lipoprotein (HDL)-associated enzyme, which catalyses hydrolyses of a broad spectrum of substrates, including organophosphorus compounds as well as oxidised lipids in the form of lipid hydroperoxides generated on low density lipoproteins (LDL). Paraoxonase is released into the blood stream and is considered a biomarker of liver function in dairy cows and is drastically reduced in chronic liver damage (Bionaz et al., 2007). Turc et al. (2005) found lower paroxonase activity in cows with hepatomegaly compared to clinically healthy cows. In this study, paroxonase was significantly affected by parity and was positively correlated to DIM and negatively correlated to EBVf; furthermore, paroxonase was positively related to EBVp in IH cows.

It is well known that negative energy balance at the beginning of lactation results in the release of a large amount of NEFA from adipose tissue, with an increased production of ketone bodies and lipids accumulation in the liver, leading to lover steatosis. However, the cows samples in the study were in mid lactation and is this situation the different paroxonase contents in plasma are more likely related to the genetic background of the animals than to the nutritive balance.

Cortisol concentration in milk and hair were not affected by breed and EBVs, and only in hair its concentration was significantly different between farms and parity and linearly related to DIM.

Table 5. Effects of breed, DIM and genetic merit (EBVp) on BCS score, milk yield and its composition, somatic cell count, urea and cortisol contents in Italian Holstein (IH) and Italian Simmental (IS) lactating cows, sampled in 5 commercial farms in North-East of Italy involved in study1.

Items	Breeds		SEM	Effects				
	IH	IS		Breed	Farm	Covariates		
						DIM	EBVpIH	EBVpIS
BCS, Score	2.75	3.14	0.03	***	***	* (+)	NS (-)	* (-)
Milk output, kg/d	35.34	30.98	4.98	***	***	** (-)	** (+)	** (+)
Milk								
Fat	1.33	1.16	0.02	**	***	* (-)	* (+)	* (+)
Protein	1.10	1.08	0.02	NS	***	* (-)	** (+)	** (+)
Milk composition, %								
Fat	3.79	3.78	0.06	NS	*	NS (+)	NS (+)	NS (-)
Protein	3.12	3.49	0.02	***	***	** (+)	** (+)	NS (+)
SCC, Count	4.22	5.05	0.13	***	***	** (+)	NS (+)	NS (-)
Urea, mmol/l	17.10	23.30	3.67	***	***	NS (-)	NS (-)	NS (+)

IH = Italian Holstein; IS = Italian Simmental; DIM = Days In Milk; EBVpIH: Estimated Breeding Values for protein of Italian Holstein cows; EBVpIS: Estimated Breeding Values for protein of Italian Simmental cows; *, **, *** Indicate significant effects ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively); NS = Not Significant effect; (+) = the covariates for the variable is positive; (-) = the covariates for the variable is negative; BCS = Body Condition Score; SCC = Somatic Cell Count

Table 6. Effects of breed, DIM and genetic merit (EBVp) on plasma and blood parameters in Italian Holstein (IH) and Italian Simmental (IS) lactating cows, sampled in 5 commercial farms in North-East of Italy involved in study 1.

Items	Breeds		SEM	Effects				
	IH	IS		Breed	Farm	Covariates		
						DIM	EBVpIH	EBVpIS
Total protein, g/l	83.6	80.6	6.0	NS	***	NS (-)	NS (+)	NS (+)
Albumin, g/l	37.5	37.2	2.5	NS	***	NS (-)	NS (+)	NS (+)
Urea, mmol/l	4.8	5.0	0.1	NS	***	* (-)	NS (-)	NS (-)
Creatinine, μmol/l	63.2	90.0	8.3	***	***	NS (+)	NS (+)	NS (-)
Zinc, μmol/l	12.9	11.2	0.2	***	**	NS (+)	NS (-)	NS (+)
TAS, mmol/l	1.2	1.1	0.1	***	NS	NS (+)	NS (+)	NS (+)
GPx, U/gHb	326	243	52	***	***	NS (+)	NS (-)	NS (+)
Glucose, mmol/l	3.40	3.40	0.02	NS	***	NS (+)	NS (+)	* (-)
NEFA, meq/l	0.12	0.13	0.01	NS	**	NS (+)	NS (+)	NS (-)
BOHB, mmol/l	0.58	0.55	0.01	NS	***	NS (+)	* (-)	* (-)

IH = Italian Holstein; IS = Italian Simmental; DIM = Days In Milk; EBVpIH: Estimated Breeding Values for protein of Italian Holstein cows; EBVpIS: Estimated Breeding Values for protein of Italian Simmental cows; *, **, *** Indicate significant effects ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively); NS = Not Significant effects; (+) = the covariates for the variable is positive; (-) = the covariates for the variable is negative; TAS = Total Antioxidant Status; GPx = Glutathione Peroxidase; NEFA = Non-Esterified Fatty Acids; BOHB = β-hydroxybutirate.

Table 7. Effects of breed, DIM and genetic merit (EBV) on urine parameters in Italian Holstein (IH) and Italian Simmental (IS) lactating cows, sampled in 5 commercial farms in North-East of Italy involved in study 1.

Items	Breeds		SEM	Effects				
	IH	IS		Breed	Farm	Covariates		
						DIM	EBVpIH	EBVpIS
Nitrogen fractions, g/l								
Total N	6.52	7.94	3.54	**	***	NS (-)	NS (-)	NS (+)
Urea N	3.96	3.44	4.30	NS	NS	NS (-)	NS (-)	NS (-)
Creatinine N	0.90	1.35	0.03	***	**	NS (+)	NS (+)	NS (+)
PD N	0.87	0.83	0.02	NS	NS	NS (+)	NS (+)	NS (-)
Ratios, unit								
Creatinine N:Total N	0.15	0.17	0.01	***	***	* (+)	NS (+)	NS (-)
Urea N:Total N	0.65	0.45	0.03	NS	***	NS (+)	NS (-)	* (-)
PD N:Total N	0.14	0.11	0.01	*	***	NS (+)	NS (+)	* (-)
PD N : Creatinine N	0.97	0.61	0.06	***	NS	NS (-)	* (-)	NS (-)

IH = Italian Holstein; IS = Italian Simmental; DIM = Days In Milk; EBVpIH: Estimated Breeding Values for protein of Italian Holstein cows; EBVpIS: Estimated Breeding Values for protein of Italian Simmental cows; *, **, *** Indicate significant effects ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively); NS = Not Significant effects; (+) = the covariates for the variable is positive; (-) = the covariates for the variable is negative; N = Nitrogen; PD = Purine Derivatives.

Table 8. Effects of breed, farm, parity, DIM and estimated breeding value for fat (EBVf), protein (EBVp) and SCC (EBVc) on BCS score, milk yield and its composition, somatic cell count, urea and cortisol contents in Italian Holstein (IH) and Italian Simmental (IS) lactating cows, sampled in 10 commercial farms in North-East of Italy involved in study 2.

Items	Breeds		Main effects and covariates									
			Main Effects		Covariates							
	IH	IS	Breed	Farm	Parity	DIM	EBVf IH	EBVf IS	EBVp IH	EBVp IS	EBVc IH	EBVc IS
BCS	2.32	3.00	ns	ns	***	***	ns(-)	ns(-)	ns(-)	ns(-)	ns(-)	ns(-)
Milk output, kg/d	35.58	26.40	ns	ns	***	***	*(-)	**(+)	***(+)	*(+)	ns(+)	*(+)
Fat, %	3.62	3.77	ns	ns	*	***	***(+)	***(+)	***(-)	***(-)	ns(-)	ns(-)
Protein, %	3.17	3.52	ns	ns	ns	***	**(+)	ns(-)	ns(+)	ns(+)	*(-)	ns(-)
Casein, %	2.50	2.76	ns	ns	ns	***	*(+)	ns(-)	ns(+)	ns(+)	ns(-)	ns(-)
Fat, kg/d	1.27	0.98	ns	ns	***	***	***(+)	***(+)	*(+)	ns(-)	ns(+)	ns(+)
Protein, kg/d	1.12	0.92	ns	ns	***	***	ns(-)	**(+)	***(+)	**(+)	ns(+)	ns(+)
Urea, mmol/l	23.63	19.92	ns	*	ns	ns	ns(+)	ns(+)	ns(-)	ns(-)	*(+)	ns(+)
SCC	3.45	3.59	ns	ns	***	ns	*(+)	ns(-)	ns(-)	ns(-)	***(+)	***(-)

IH = Italian Holstein; IS = Italian Simmental; DIM = Days In Milk; EBVfIH, EBVpIH, EBVcIH: Estimated Breeding Values for fat, protein and somatic cell count of Italian Holstein cows; EBVfIS, EBVpIS, EBVcIS,: Estimated Breeding Values for fat, protein and somatic cell count of Italian Simmental cows; *, **, *** Indicate significant effects ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively); NS = Not Significant effect; (+) = the covariates for the variable is positive; (-) = the covariates for the variable is negative; BCS = Body Condition Score; SCC = Somatic Cell Count

Table 9. Effects of breed, farm, parity, DIM and estimated breeding value for fat (EBVf), protein (EBVp) and SCC (EBVc) on plasma and blood parameters in (IH) and Italian Simmental (IS) lactating cows, sampled in 10 commercial farms in North-East of Italy involved in study 2.

Items	Breeds		Main effects and covariates									
			Main Effects			Covariates						
	IH	IS	Breed	Farm	Parity	DIM	EBVf IH	EBVf IS	EBVp IH	EBVp IS	EBVc IH	EBVc IS
Cholesterol, mmol/l	5.89	4.86	*	ns	***	ns	*(-)	ns(+)	**(+)	ns(+)	ns(-)	ns(+)
Urea, mmol/l	5.48	4.70	ns	*	ns	**	ns(+)	ns(+)	**(-)	*(-)	*(+)	ns(+)
AST/GOT, U/L)	101.20	90.69	ns	ns	ns	ns	*(-)	ns(+)	**(+)	ns(-)	ns(-)	ns(-)
gGT, U/L	32.67	28.37	ns	ns	**	**	ns(-)	ns(+)	***(+)	ns(-)	ns(-)	ns(+)
NEFA, mmol/l	0.12	0.10	ns	ns	ns	***	ns(-)	ns(+)	ns(+)	ns(+)	ns(+)	ns(+)
BOHB, mmol/l	0.68	0.69	ns	ns	ns	**	***(+)	***(+)	**(-)	*(-)	ns(+)	ns(+)
Creatinine, µmol/l	87.11	111.60	**	ns	ns	**	ns(-)	*(-)	ns(+)	ns(+)	ns(+)	ns(+)
Bilirubine, µmol/l	0.74	1.09	ns	ns	***	***	ns(-)	ns(-)	ns(+)	ns(+)	ns(+)	ns(-)
Glucose, mmol/l	3.82	3.90	ns	ns	ns	ns	**(-)	*(-)	ns(+)	ns(+)	ns(-)	ns(-)

IH = Italian Holstein; IS = Italian Simmental; DIM = Days In Milk; EBV fIH, EBVpIH, EBVcIH: Estimated Breeding Values for fat, protein and somatic cell count of Italian Holstein cows; EBVfIS, EBVpIS, EBVcIS,: Estimated Breeding Values for fat, protein and somatic cell count of Italian Simmental cows; *, **, *** Indicate significant effects ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively); NS = Not Significant effect; (+) = the covariates for the variable is positive; (-) = the covariates for the variable is negative; NEFA = Non-Esterified Fatty Acids; BOHB = β-hydroxybutirate, AST/GOT= Aspartate aminotransferase/ Serum glutamic oxaloacetic transaminase; gGT=Gamma-glutamyl transferase,

Table 10. Effects of breed, farm, parity, DIM and estimated breeding value for fat (EBVf), protein (EBVp) and SCC (EBVc) on proteins, acute-phase proteins and zinc in plasma and on milk and hair cortisol in Italian Holstein (IH) and Italian Simmental (IS) lactating cows, sampled in 10 commercial farms in North-East of Italy involved in study 2.

Items	Breeds		Main effects and covariates									
			Main Effects			Covariates						
	IH	IS	Breed	Farm	Parity	DIM	EBVf IH	EBVf IS	EBVp IH	EBVp IS	EBVc IH	EBVc IS
Total Protein, g/l	80.26	78.31	ns	ns	***	ns	ns(+)	*(+)	ns(+)	ns(+)	ns(+)	*(+)
Albumin, g/l	36.76	37.43	ns	ns	***	ns	ns(+)	ns(+)	ns(+)	ns(+)	ns(+)	*(+)
Globulin, g/l	43.49	40.87	ns	ns	***	ns	ns(+)	ns(+)	ns(-)	ns(+)	ns(+)	**(+)
Zinc, µmol/l	13.23	12.78	ns	ns	*	**	ns(+)	ns(-)	ns(+)	ns(-)	ns(-)	ns(-)
Ceruloplasmin, mmol/l	2.61	2.57	ns	*	**	ns	ns(+)	ns(-)	ns(-)	ns(+)	ns(-)	ns(-)
Haptoglobin, g/l	0.43	0.36	ns	ns	*	ns	ns(+)	*(-)	ns(-)	ns(-)	ns(-)	ns(-)
Paraoxonase, U/ml	103.00	101.10	ns	ns	***	***	***(-)	ns(-)	***(+)	ns(+)	ns(+)	ns(+)
Cortisol milk, ln(ng)/ml	6.09	6.11	ns	ns	*	ns	ns(+)	ns(-)	ns(+)	*(+)	ns(+)	ns(+)
Cortisol hair, ln(ng)/g	1.20	1.03	ns	*	***	**	ns(+)	ns(+)	ns(-)	ns(-)	ns(+)	ns(-)

IH = Italian Holstein; IS = Italian Simmental; DIM = Days In Milk; EBV fIH, EBVpIH, EBVcIH: Estimated Breeding Values for fat, protein and somatic cell count of Italian Holstein cows; EBVfIS, EBVpIS, EBVcIS,: Estimated Breeding Values for fat, protein and somatic cell count of Italian Simmental cows; *, **, *** Indicate significant effects ($P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively); NS = Not Significant effect; (+) = the covariates for the variable is positive; (-) = the covariates for the variable is negative; lnHCm= natural logarithm of cortisol concentration in milk; ln HCh== natural logarithm of cortisol concentration in hair

Considerations

Our results propose that biomarkers of metabolism measured in blood, urine and milk contain information that can be partly related to the breed and to the genetic merit, other than to environmental factors. Of particular interest, biomarkers of energy metabolism, as plasma BOHB, glucose, are more related to EBVs than biomarkers related to immune response, as ceruloplasmin, haptoglobin, globulin, albumin and zinc.

Further studies are needed to ascertain the weight of genetic component on these metabolites and if they can be used as predictors of metabolic efficiency of dairy cows.

Part 2.

Reinterpretation of cortisol in milk, blood and hair for the evaluation of animal welfare and health in Holstein and Simmental cows

Results

According to the results of part 1, biomarkers of HPA axis stimulation and immune response were fairly associated to breed and EBVs, suggesting a predominant influence of environmental conditions on genetic background. To further investigate if these biomarkers are related to animal well being or health, cows were classified on the basis of SCC in the milk and a different statistical model was applied.

The partitioning of cows in the 3 classes of SCC in the 10 farms is reported in Table 11. The vast majority of the cows were included in class 1, indicating the health condition of mammary gland and about 20% of the population was assigned to class 3.

The effect of breed, class of SCC and farm on cortisol concentration in milk and plasma in study 1 is reported in Table 12. The data refer to 4 of the 5 farms involved in this study, and farm C was excluded in this analysis, to balance cows between breeds. Cortisol content in milk was higher in IH than IS cows ($P < 0.05$), whereas plasma cortisol did not differ between breeds. Differences for milk and plasma cortisol concentrations between classes of SCC were not significant, while significant effect was shown between farms ($P < 0.01$). No significant effects of covariates DIM and milk yield were observed. Cortisol content in milk was not correlated to plasma content (Figure 1, $r = 0.109$, $P > 0.05$) in study 1 and the mean milk to plasma cortisol ratio was about 1:30.

The effect of breed, class of SCC and farm on cortisol concentration in milk and hair in study 2 is reported in Table 13. The values of cortisol content in hair were higher in IH than IS cows ($P < 0.05$) like in the study 1. Differences for milk and hair cortisol concentrations between classes of SCC were significant, and class 3 of SCC showed the highest concentration of cortisol ($P < 0.05$). Significant effect was shown between farms ($P < 0.01$), but cortisol content in milk was not correlated to hair content (Figure 2).

The effect of breed, class of SCC and farm on protein, acute phase protein and zinc in plasma is reported in Table 14. The values of BCS, zinc, ceruloplasmin and albumin were higher in IS cows compared to IH cows, while total protein, globulin, haptoglobin and paraoxonase were high in IH cows than IS cows. ($P < 0.05$). Significant differences for BCS, plasma ceruloplasmin, total protein, albumin, globulin, haptoglobuli and paraoxonase between classes of SCC was observed ($P < 0.05$). Also significant effect between farms was shown ($P < 0.01$).

Discussion

The aim of the first study was to compare milk, blood and hair cortisol concentrations and to consider its variability in milk in relation to farm, milk yield and DIM. In the model, also 3 classes of SCC and breed were included, even though the results have to be considered with caution since only 20% of cows were sampled. The classification criteria for SCC were more than 200,000 SCC/ml, a threshold for inflammatory response indicating that a subclinical mastitis is occurring (Hillerton, 1999), and more than 400,000 SCC/ml, the upper limit indicated by the European Union for human consumption (Europa, 2009).

Since data of cortisol in milk and plasma deviated from normality, the natural logarithm transformation was required before the statistical analysis. The overall mean milk cortisol concentration in study 1 was 330 pg/mL for the untransformed values (298 pg/mL for the exponential of log transformed values) and lies within the range of values reported by Verkerk et al. (1998) and Fukasawa & Tsukada (2010). Also the concentration of serum cortisol were within the

ranges reported by Gabai et al. (2006). Milk and blood cortisol concentrations were not correlated (Figure 1), probably reflecting the episodic secretion of cortisol in blood sampled from restrained animals. These result do not agree with those of Gygax et al. (2006), who reported that measurements of cortisol concentrations in milk and blood correlate closely. Also Schutt & Fell (1985) and Verkerk et al. (1998) reported a correlation between serum and milk cortisol, but the data obtained by these authors referred to free fraction of blood and milk cortisol (Schutt & Fell, 1985) and were obtained from animals sampled after severe adrenal stimulation. Moreover, Verkerk et al. (1998) suggested that milk cortisol can reflect serum concentration only within 2-4 hours after the response to acute stressors of lactating cows. According to Fox et al. (1981), adrenal cortisol secretion is transferred from blood to milk rapidly (within 4 h), but in the absence of sustained activation of HPA axis this transfer decline and cortisol in diluted later, as a function of milk yield and milking interval. Therefore, considering that in our trial cows were milked about every 12 hours, cortisol concentration in milk likely represents a picture of the average blood cortisol variations in the previous 10 to 14 hours window. It must also be considered that in our study the collection of blood was subsequent to milk sampling, the first referring to the acute secretion and the latter to the previous 12 hours secretion. However, the aim of the comparison was not only to study the correlation between sampling site but also to investigate if the breed and farm effects were similar using the two biological fluids. As can be seen from Table 12, the statistical effect of breed or farm factors differed from plasma to milk cortisol contents.

The difference of cortisol content in milk observed between breeds in the first study (Table 12) can be ascribed to the different ability to cope with milk yield for IH and IS. High yielding cows (IH) are selected for milk production whilst IS cows are dual purpose animals (www.anafi.it; www.anapri.it), (i.e. milk and meat production). The higher values of milk production observed in the present study for IH cows (Table 12) can arise from the catabolic activity of glucocorticoids (Elasser et al., 2000), as can be further supported by the positive relationship between milk cortisol and milk yield. Higher serum cortisol in Holstein in comparison the Montbeliarde-sired crossbred

cows were reported by Mendonça et al. (2013) during the transition period. Also Negrao & Marnet (2006) found that Holstein cows with higher milk yield had higher levels of plasma cortisol. In this experiment, the authors sampled the cows 7 consecutive days using a catheter implanted into the jugular vein 72 hours before the first sampling to avoid acute cortisol secretion linked to animal handling.

However, the more productive IH cows of FD showed the highest milk cortisol, but also the least productive IS cows of FA had significant ($P < 0.01$) higher milk than cows of FB and FC. It is well known that individual differences in response to stress are affected by both genetic (Burrow 1997) and environmental factors.From the results obtained in the present study, different rearing conditions, such as stocking rate, shape and type of cubicles and number of animals within the productive groups, confirm that the effect of environment on HPA axis can superimpose the genetic background (Diaz, 2013).

The effects of breed, class of SCC and farm on milk cortisol levels in the second study was investigated in a larger number of cows and farms (Table 5), with the aim to validate the results obtained in the pilot study. The effect of breed on milk cortisol concentration was the same evidenced in study 1, strengthening the hypothesis that IH cows have a higher activation of HPA axis to cope with the higher milk yields. A significant ($P<0.01$) effect of farms was calculated, confirming that the environment plays a relevant role in determining the milk cortisol concentration and has thus to be taken into consideration. A significant effect of SCC class on milk cortisol ($P < 0.05$), with higher levels of milk cortisol in class 3 was also calculated. Previous studies conducted both on cows and goats did not find correlation between milk cortisol concentration and the level of SCC (Diaz et al., 2013). However, according to Diaz et al., (2013), if severe stress occurs, the concentration of cortisol in blood increases and SCC increases. Our results obtained from a larger number of lactating cows, suggest that milk cortisol concentration provides information on mammary gland health complementary to environment and breeds. It is likely that, in the presence

of sustained activation of HPA axis for a period longer than 2-4 hours, milk cortisol reflects the blood level.

In study 2, cortisol was analyzed also in white hair sampled from the tail. There are a lot of studies that report the presence of corticotropin releasing hormon (CRH) and of proopiomelanocortin (POMC) derived ACTH, as well as of the corresponding receptors in hair follicles (Slominski et al.,1999; Roloff et al., 1998; Kauser et al., 2006). These studies established the hair follicle as a prominent source and a potential target for the bioactivity of POMC products, suggesting a role for POMC-derived peptides in maintaining the immune privilege properties of the anagen hair follicle (Paus, 1999). A high concentrations of cortisol in hair have a positive correlation with chronic stress found in humans and animals in different clinical situation, such as acute myocardial infarction in men (Pereg et al., 2011), chronic pain in adults (Van Uum et al., 2008) and Cushing's syndrome (Thomson et al., 2010). Elevated levels of circulating glucocorticoids exert a variety of catabolic, antireproductive, antigrowth, and immunosuppressive effects that mobilize and repartition energy to help organisms restore homeostatic balance (Habib et al. 2001; Charmandari et al. 2005).

In some studies, hair cortisol is considered to represent serum cortisol, that has been transmitted from the bloodstream to the hair follicle (Davenport et al., 2006 ; Accorsi et al., 2008 ; Koren et al., 2002). However, in our study there was not correlation between milk and hair cortisol (Figure 2), probably because the hair cortisol reflected the long period exposure of stress. The production of cortisol in hair could be independent of central HPA influences in similar ways to its production in cultured melanocytes (Slominski et al., 2005) and dermal fibroblasts. Ito et al.'s (2005) report of the independence of the hair follicle as a producer of cortisol challenges that perspective and leaves open the future resolution of the original source of hair cortisol as systemic or of local origin.

In study 2 we collected only white hair from the tail of two breeds. In literature was reported that concentration of cortisol in hair change based on different factors, as age, sex and region of the body. Hair grows fastest on the vertex region of the scalp, with the largest percent of follicles in the

anagen stage. Scalp hair grows faster than pubic hair (Harkey, 1993) and women's scalp hair grows faster than that on men (Saitoh et al., 1969). Macbeth et al. (2010) found that hair collected from the neck of grizzly bears had significantly higher cortisol concentrations than hair collected from the shoulder, rump, and abdomen, research with beef cattle has shown that cortisol concentrations were highest when hair was collected from the tail switch (Moya et al., 2013). It has also been reported that difference in cortisol concentrations depends from the color of the hair. White hair had greater concentrations of cortisol than black hair (Gonzálezde-la-Vara et al. 2011).Similarly in a dog studies, it has been reported that yellow hair had greater concentrations of cortisol than and black hair from German Shepherds (Bennett and Hayssen, 2010). Reasons for this difference are not well understood but could be related to mechanisms associated with melanocyte development (Slominski et al., 2005), differentiation (Roulin et al., 2008), or simply a question of physical space within the hair shaft, as white or yellow hair have less pigmentation.

A significant effect on cortisol concentration in hair was observed for parity and a positive correlation between hair cortisol concentration and age in infant rhesus macaques has been shown (Dettmer et al., 2011).

A significant effect of class of SCC on hair cortisol and milk cortisol ($P < 0.05$), with higher levels in class 3 was observed, suggesting that at some extent cortisol in hair or in milk is an index of central HPA axis activity. This suggestion is confirmed by the mean value of acute phase proteins between classes of SCC. The relationship between inflammation and general health conditions in dairy cows and the link between inflammation, liver function, fertility and metabolic response is well known. Inflammations are not always associated with clinical disease, but often can be discovered only by specific blood indices. The major effect of cytokines in liver is the stimulation of acute phase response (Fleck, 1989), which is characterized by induction of positive acute phase protein (+APP) synthesis, mostly detected in blood plasma (e.g., haptoglobin and ceruloplasmin) and the impairment of hepatic synthesis of negative acute phase proteins (−APP), such as albumin and retinol binding protein. The proinflammatory cytokines stimulate glucocorticoid's release,

reduce feed intake (Johnson and Finck, 2001) and increase energy expenditures (Elsasser et al., 2000). A rule of a negative interference of cytokines in the biological activities are known and Barton et al. (1996) reported that excess crude protein (high blood urea) reduces the reproductive efficiency only in cows with major health problems. The effect of glucocorticoids on tissue priority for nutrients (Elsasser et al., 2000) is a diversion of liver synthesis that can exacerbate the negative energy balance and increase the risk of liver lipidosis (Katoh, 2002), reducing reproductive efficiency (Butler, 2000). The significant higher value of ceruloplasmin and haptoglobin in the 3 class of SCC agrees with the primary function of haptoglobin, that is a prevention of the loss of iron by the formation of very stable complexes with free hemoglobin in the blood. Consequently, haptoglobin has a bacteriostatic effect by restricting the availability of iron necessary for bacterial growth.

Table 11. Partitioning of cows in the 3 classes of SCC between the 10 commercial farms involved in study 2.

	Farm										Total
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	
Class 1	110	60	51	102	49	84	60	44	65	77	702
Class 2	31	11	15	11	7	3	25	9	4	8	124
Class 3	38	17	26	15	19	5	40	8	5	25	198
Total	179	88	92	128	75	92	125	61	74	110	1024

Class 1 = SCC ≤ 200,000/ml:
Class 2 = 200,001/ml < SCC ≤ 400,000/ml
Class 3 = SCC > 400,001/ml:

Table 12. Effect of breed, class of somatic cell counts, farm on cortisol concentration in milk and plasma of dairy cows in study 1. The model includes the linear effect of days in milking (DIM) and milk yield (Milk, kg/d).

	Milk			Plasma		
	Mean	s.e.		Mean	s.e.	
Breed						
IH	5.77	0.06	a	7.81	0.11	Ns
IS	5.62	0.07	b	8.02	0.08	Ns
Class						
1	5.72	0.05	Ns	7.84	0.09	Ns
2	5.55	0.15	Ns	8.01	0.16	Ns
3	5.70	0.05	Ns	7.90	0.07	Ns
Farm						
A	5.83	0.08	A	8.24	0.10	A
B	5.44	0.09	B	7.84	0.11	B
C	5.61	0.11	B	7.27	0.12	C
D	5.92	0.06	A	8.31	0.13	A
DIM	-0.002	0.001	Ns	-0.001	0.002	Ns
Milk	0.010	0.007	Ns	-0.006	0.011	Ns
General Mean	5.72	0.06		7.98	0.09	

IH: Italian Holstein; IS: Italian Simmental. Covariates appearing in the model are evaluated at the following values for Milk and Plasma cortisol of experiment 1: DIM = 142.3; Milk = 33.6
a, b = P < 0.05
A, B,C = P < 0.01
NS = Not Significant effects

Table 13. Effect of breed, class of somatic cell counts and farm on cortisol concentration in milk and in hair of dairy cows in study 2. Data are reported as estimated means of natural logarithm of cortisol

	Milk Cortisol			Hair Cortisol		
	Mean	se		Mean	se	
Breed						
IH	6.43	0.07	A	1.25	0.06	A
IS	5.94	0.06	B	1.03	0.04	B
Class						
1	6.15	0.02	b	1.11	0.02	b
2	6.14	0.05	b	1.13	0.04	b
3	6.27	0.04	a	1.18	0.03	a
Farm						
10	6.05	0.05	B	1.67	0.04	A
5	6.19	0.06	AB	1.05	0.05	B
1	6.02	0.04	B	0.50	0.03	C
4	6.10	0.05	AB	1.56	0.04	AB
3	6.33	0.06	A	0.99	0.05	B
2	6.04	0.06	B	0.82	0.05	BC
6	5.85	0.06	C	1.21	0.04	B
8	6.36	0.07	A	1.31	0.05	B
7	6.37	0.05	A	0.46	0.04	C
9	6.01	0.06	B	1.48	0.05	AB
DIM	0.001	0.001		0.006	0.002	
Milk	0.001	0.003		0.421	0.003	
General mean	6.19	0.03		1.14	0.02	

Covariates appearing in the model are evaluated at the following values: DIM = 160.07, latte_kg_d = 31.192
IH: Italian Holstein; IS: Italian Simmental
a, b = P < 0.05
A, B, C = P < 0.01
NS = Not Significant effects

Table 14. Effect of breed, class of SCC and farm on in acute phase protein of dairy cows in study 2.

	BCS			Zinc			Ceruloplasmin			Total Protein			Albumin			Globulin			Haptoglobin			Paraoxonase		
	Mean	se		Mean	se		Mean	se		Mean	se		Mean	se		Mean	se		Mean	se		Mean	se	
Breed																								
IH	2.11	0.05	b	12.77	0.40	Ns	2.57	0.07	Ns	81.47	0.75	a	35.98	0.32	b	45.48	0.80	a	0.51	0.04	Ns	101.90	2.90	Ns
IS	3.09	0.03	a	12.88	0.31	Ns	2.72	0.05	Ns	79.31	0.59	b	37.55	0.25	a	41.75	0.62	b	0.39	0.03	Ns	99.29	2.27	Ns
Class																								
1	2.64	0.01	a	12.98	0.13	a	2.47	0.02	c	78.01	0.25	c	37.29	0.10	a	40.72	0.26	c	0.34	0.02	a	103.80	0.98	a
2	2.64	0.03	a	12.78	0.30	a	2.59	0.05	b	79.91	0.55	b	37.16	0.23	a	42.74	0.58	a	0.43	0.03	b	101.50	2.16	a
3	2.52	0.03	b	12.71	0.24	a	2.88	0.04	a	83.24	0.45	a	35.84	0.19	b	47.40	0.47	b	0.58	0.03	a	96.476	1.77	b
Farm																								
10	2.89	0.05	AB	13.39	0.45	B	3.05	0.08	A	79.80	0.85	AB	38.37	0.35	A	41.43	0.89	B	0.45	0.05	B	97.41	3.28	AB
5	2.77	0.06	BC	12.28	0.51	B	2.97	0.09	AB	80.15	0.94	AB	35.96	0.40	BC	44.18	1.00	AB	0.46	0.05	B	99.11	3.67	AB
1	2.97	0.05	A	14.72	0.43	AB	2.98	0.07	AB	76.82	0.80	C	37.16	0.33	AB	39.65	0.84	C	0.47	0.04	B	108.10	3.10	A
4	2.78	0.05	B	12.32	0.48	B	2.21	0.08	B	82.42	0.90	A	36.60	0.38	B	45.82	0.95	A	0.45	0.05	B	93.75	3.47	B
3	2.41	0.05	C	16.10	0.46	A	1.58	0.08	C	79.85	0.87	AB	38.15	0.36	A	41.69	0.92	B	0.56	0.05	A	102.50	3.37	AB
2	2.28	0.05	C	11.90	0.47	BC	2.39	0.08	B	82.05	0.88	A	37.76	0.37	AB	44.29	0.92	AB	0.51	0.05	AB	90.31	3.39	B
6	2.38	0.05	C	11.24	0.45	C	2.83	0.08	AB	82.38	0.85	A	35.50	0.35	BC	46.88	0.89	A	0.48	0.05	B	113.40	3.30	A
8	2.02	0.06	D	11.28	0.53	BC	2.83	0.09	AB	80.15	0.98	AB	35.62	0.41	BC	44.52	1.03	AB	0.35	0.06	BC	113.30	3.81	A
7	2.60	0.04	BC	12.23	0.37	B	3.16	0.06	A	82.27	0.69	A	36.75	0.29	AB	45.51	0.73	A	0.32	0.04	C	89.28	2.68	B
9	2.91	0.06	AB	12.75	0.50	B	2.48	0.09	B	78.00	0.94	B	35.78	0.39	BC	42.21	0.99	B	0.44	0.05	B	98.66	3.65	AB
DIM	0.000			0.002			0.726			0.637			0.016			0.159			0.004			0.003		
Milk	0.000			0.934			0.003			0.083			0.000			0.542			0.000			0.050		
General mean	2.6	0.018		12.82	0.14		2.65	0.02		80.39	0.27		36.76	0.11		43.62	0.28		0.45	0.01		100.60	1.05	

IH: Italian Holstein; IS: Italian Simmental; a, b, c = $P < 0.05$, A, B, C, D = $P < 0.01$; NS = Not Significant effects

Figure 1. Correlation between milk and plasma cortisol (ln of pmol/ml) concentrations (r = 0.109)

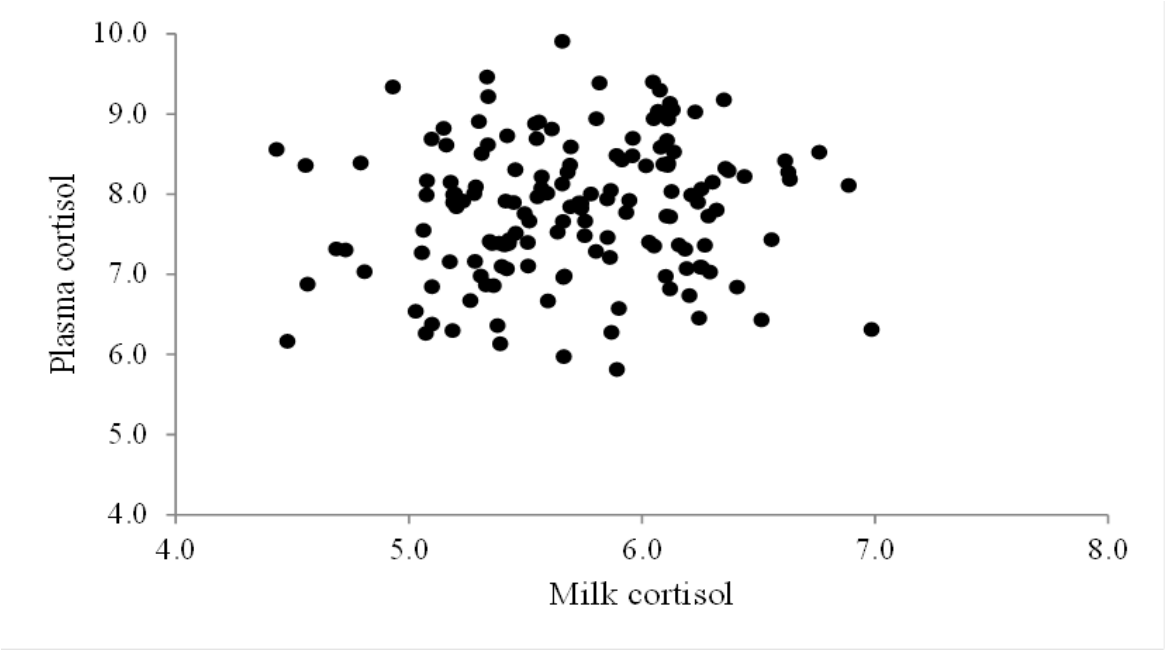
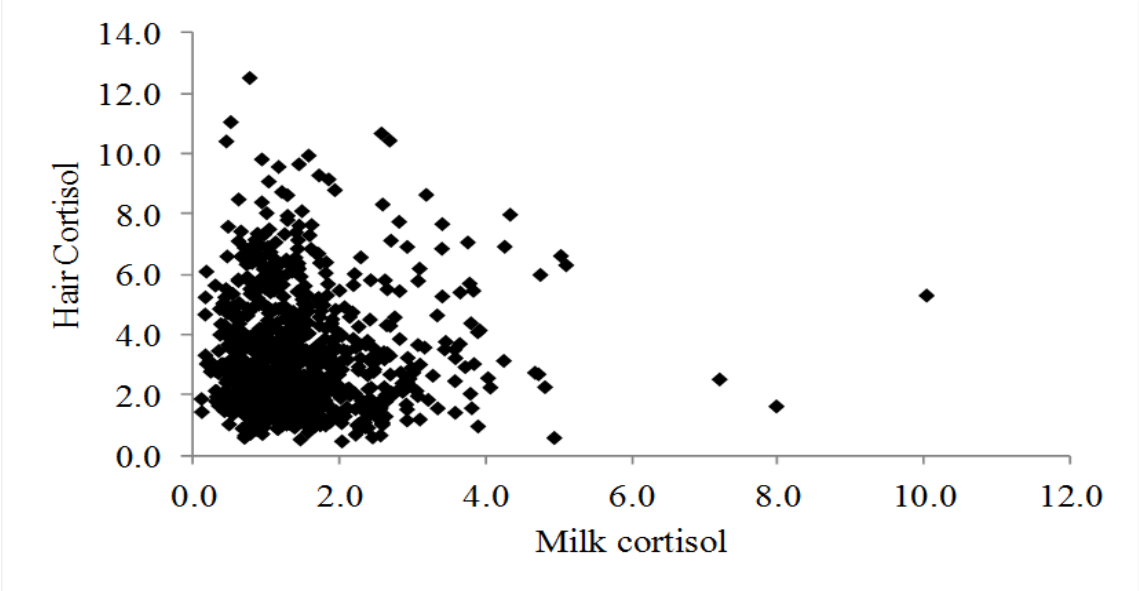


Figure 2. Correlation between milk (mmol/ml) and hair (pg/g) cortisol concentrations in study 2 (r = -0.01)



Considerations

These original results on milk and hair cortisol deserve further studies to investigate the physiological basis of the relationship with breed, milk yield and SCC, to ascertain the reliability of cortisol in milk or hair as a biomarker of healthy status of mammary gland. Moreover, a study of time course analysis of milk cortisol for several days could assist farmers and technicians to evaluate chronic conditions of animals. The concordance of milk cortisol with the acute phase protein in blood confirms the usefulness of the analysis of this hormone to depict the health condition and well being of the cow at a farm level.

The advantages of using milk samples arises from the easy to collect them, since can be obtain directly in milking parlour without animal handling and overcomes some of the problems associated with other sampling sites, as blood, urine and faeces. For this reason, milk can be considered a preferential site of sampling in dairy cows to point out short term stimulation of the HPA axis.

Conclusions

The data presented in this thesis were collected in a large number of lactating cows reared in commercial farms in a homogenous conditions in North East of Italy. It is often reported that the process of selection for productive traits leaded to a less robust cows and for this reason, the main aim of the study was to investigate if cows of variable genetic merit shows a different ability to respond to the environmental stimuli, that they found in real conditions, i.e. in commercial farms. Samples were collected after the peak of lactation and the so call transition period, to avoid metabolic imbalances and other perturbations, which are often present around calving.

One criticism of the data presented arises from the fact that a single sample was collected for each cows and that definite conclusion can not be drawn, since repeated measure are considered more

solid for variables measured in biological fluids. However, the statistical approach used clearly showed that productive data (i.e. milk yield and its constituents, and SCC) were linearly related with the respective EBV, although those data originated from a unique sample.

Biomarkers of metabolism and oxidative stress were also in line with the EBVs and in particular BOHB is a promising compound to measure also in mid lactation, when subclinical ketosis is not more a potential problems for the lactating cows, since contains genetic information other than nutrition relationship.

Instead, biomarkers of animal health, as acute phase proteins in plasma and cortisol in plasma, milk or hair, are more related to environment and, probably, the complex regulatory mechanisms of these compounds does not allow to link them directly to genetic background. These biomarkers, and milk cortisol in particular, are promising compound to measure to complement the assessment of animal welfare protocols.

Legend

ACTH = Adrenocorticotropin hormone

APP = Acute phase proteins

AST/GOT = Aspartate aminotransferase/ Serum glutamic oxaloacetic transaminase

AVP = Arginine vasopressin

BCS = Body condition score

BOHB = β-hydroxybutirate

CP = Crude protein

DIM = Days in milk

DM = Dry matter

DMI = Dry matter intake

EBV = Estimated breeding values

EBVc = Estimated breeding values for somatic cell count in milk

EBVf = Estimated breeding values for milk fat

EBVp = Estimated breeding values for protein milk

EE = Ether extract

EREs = Estrogen response elements

GC = Glucocorticoids

gGT = Gamma-glutamyl transferase

GH = Growth hormone

GnRH = Gonadotropin-releasing hormone

GPx = Glutathione peroxidase

GR = Glucocorticoid receptor

GRE = Glucocorticoid responsive elements

HPA = Hypothalamic – pituitary–adrenal

IGF = Insulin-like growth factor

IH = Italian Holstein

IL-1 and IL-6 = Interleukin -1 ; Interleukin - 6

IS = Italian Simmental

LC = Locus ceruleus

ln HCh = Natural logarithm of cortisol concentration in hair

ln HCm = Natural logarithm of cortisol concentration in milk

N = Nitrogen

NDF = Neutral Detergent Fiber

NE = Noradrenergic

NEFA = Non-Esterified Fatty Acids

NEl = Net Energy for lactation.

NF = Nuclear factor

PD = Total purine derivatives

PDI = Protein digested in the small intestine

PDIE = Protein digested in the small intestine supplied by rumen undegraded dietary protein and by microbial protein from rumen-fermented organic matter

PDIN = Protein digested in the small intestine supplied by rumen undegraded dietary protein and by microbial protein from rumen degraded

POMC = Proopiomelanocortin

PVN = Paraventricular nuclei

RFI = Residual feed intake

SCC = Somatic cell count

SR = Stress resistance

TAS = Total antioxidant status

TMR = Total mixed ration

TNF-α = Tumor necrosis factor alpha

TRH = Thyrotropin-releasing hormone

TSH= thyroid-stimulating hormone

Zn= Zinc

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