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HAIR: A TOOL TO EVALUATE THE HPA AXIS ACTIVITY

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ABSTRACT

The hypothalamic-pituitary-adrenal (HPA) axis consists of a complex set of interactions between the hypothalamus, pituitary gland and adrenal cortex, which mediates the stress response through the actions of glucocorticoids (GCs).

Plasma, salivary, urinary, milk and faecal samples are routinely used to measure cortisol concentrations. Analysis in these matrices offer short-term information but poses considerable problems when aiming to derive information on long-term cortisol secretion due to the high variability of the HPA axis activity. Hair cortisol is a novel marker to measure long-term secretion cortisol free from many methodological aspects associated with the other matrices. For decades hair analysis has been successfully used in forensic science and toxicology to evaluate the exposure to exogenous substances and assess endogenous steroid hormones. Evaluation of cortisol in hair matrix began about a decade ago and have over the past five years had a remarkable development by advancing knowledge and affirming this method as a new and efficient way to study the HPA axis activity over a long time period.

The analysis of cortisol in hair in relation to the study of the HPA axis has been mainly evaluated in humans or nonhuman primates and no studies were developed in livestock. In farm animals, certain environmental or management conditions can potentially activate the HPA axis.

Given the importance of cortisol in monitoring the HPA axis activity, a first approach has involved the study on the distribution of hair cortisol concentrations in healthy dairy cows giving a physiological range of variation of this hormone. In a range of values from 0.8 to 20.41 pg/mg, 50% of the evaluated animals showed hair cortisol concentrations lower than 3.13 pg/mg. A change in environmental conditions, such as the movement of cows from indoor winter farming to summer grazing pastures, has led to a significant increase ($P<0.01$) in hair cortisol concentrations. However, all the mean hair cortisol concentrations recorded were comprised in the range described previously for healthy lactating dairy cows. The limited increase in the cortisol levels indicated that the transition from winter housing to summer grazing did not activate so markedly the HPA axis in the animals. It is likely that on the farms the transition feed used, the low stocking density and the concentrate offered to animals during summer grazing helped the cows adapt to the mountain pasture conditions. In addition, the greater space of the

pastures may reduce the stress of social mixing or hierarchy in dairy cows. Significant increases were also recorded in cows clinically compromised that had recently suffered a disease or physiologically compromised than cows clinically normal. A significant positive correlation ($P<0.001$) was detected between hair cortisol and cows clinically or physiologically compromised. Increase in cortisol concentration in animals clinically and physiologically compromised seems to suggest that these cows were subjected to repeated HPA axis activation. These long-term changes in basal cortisol secretion could affect homoeostasis leading to an increased susceptibility to several diseases. An individual trend in the hair cortisol concentrations has been observed in Holstein Friesian heifers compared with heifers Crossbreed F1, pointing out that the response of an organism to maintain allostasis can be poorly predicted because of the many disturbing factors. Despite this variability, Crossbreed F1 heifers showed significantly ($P<0.01$) lower cortisol concentrations compared to pure animals, showing a higher level of resilience and a better adaptability to the environment. A breed influence has also been observed in a preliminary study on the activation of the HPA axis during the moving of young Simmental and Brown Swiss bulls from the breeding centre to the artificial insemination centre and a statistically significant lower spermatozoa concentration has been observed in those with higher hair cortisol concentrations.

Due to its characteristics of reflecting extended periods of time (months to years) and for providing retrospective information, hair proved to be an excellent matrix also in the study of the activation of the HPA axis during the transition from the prenatal, the neonatal period and early months of life in relation also to environmental factors such as temperature, rainfall and light hours.

A long-term retrospective picture can also be found in other matrices such as claw horn of calves, a category of animal that is still free from any podal disorder due to excessive weight load, dietary, environmental or production disequilibrium. Even the claws of pets can provide background information on the activation of the HPA axis during the transition from the prenatal period, the neonatal and early months of life. Unlike hair, human nails and calves claws, the claws of mature dairy cows cannot be used as a matrix to provide a retrospective image of the HPA axis activity. The claws of mature dairy cows are an active and bidirectional matrix. Given that bovine belong to the Artiodactyla order, the walking pressure, caused by an uneven load, produces an

increased blood supply that could induce a different steroid incorporation on the various claw's areas.

The hair was also an excellent matrix for the study of the HPA axis activity as a result of the relocation. The transfer of animals, their relocation and the impact of new staff dedicated to their care are factors capable of generating stress in any animal, particularly in rabbits, which are very sensitive to external stimuli and easily frightened. In accordance with this, a study showed an increased hair cortisol concentration related to an environmental change (transfer at the animal facility) and to the change of personnel at the facility pointing out the importance of a protocol for the conditioning and habituation in experimental animals.

Even though the literature on hair cortisol measurement is growing quickly, many other aspects of hair analysis still require a discussion, e.g., how the steroids are incorporated into the hair shaft and if the hair pigmentation can have an impact on the steroids concentration. It is also important to take the cortisol from the sebaceous glands and sweat glands that is deposited on the hair surface into account. Evaluate this aspect could provide new potentialities of the hair matrix. In a preliminary study cortisol measured in unwashed hair showed a comparable daily trend with salivary cortisol. Further studies are also needed to understand how long a variation of the HPA axis activity has to last to be detected in the hair. An investigation on the "biological sensitivity" of the hair cortisol measurement could be an interesting challenge in developing new studies. The question about the duration and the amount of activation of the HPA axis that is requested to be reflected in hair cortisol levels has still to be answered.

Another issue that deserves attention is the anatomical area of sampling since different growth rates of hair at different anatomical sites have been reported. A study in rabbits pointed out that rabbits kept in stable environmental conditions showed no significant differences in hair cortisol levels among the different body sites. This means that in the absence of stressful stimuli that could activate the HPA axis, hair evinced similar cortisol concentrations regardless of length. On the other hand, in situations characterized by uncontrolled environmental conditions it is important keep the hair sampling site constant across the subjects and to use the re-shaving technique to easily

overcome the issue of different growth speed and different deepness of the follicle in the skin.

The use of hair analysis in research holds great promise to significantly enhance current understanding on the role of HPA axis over a long period of time. Along with the retrospective assessment of integrated cortisol secretion over extended periods of time, hair analysis in research constitutes a highly promising method for evaluating micro and macro elements that mediate effects on health and disease.

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Figure 11. Hair cortisol levels determined at the 26 body sites in rabbits.

LIST OF ABBREVIATIONS

^{3}H	Tritium
11β-HSD	11β-hydroxysteroid dehydrogenase
11β-HSD1	11β-hydroxysteroid dehydrogenase type 1
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
AVP	Arginin vasopressin
ACTH	Adenocorticotropic hormone
BCS	Body condition score
CBG	Corticosteroid-binding globulin
CLIA	ChemiLuminescent Immuno Assay
CRH	Corticotropin-releasing hormone
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
EIA	Enzyme Immune Assay
ELISA	Enzyme Linked ImmunSsorbent Assay
F1	Filial 1
FCM	Fat corrected milk
GCs	Glucocorticoids
GREs	Glucocorticoid response elements
GRs	Glucocorticoid receptors
HPA	Hypothalamic-pituitary-adrenal
HPLC/MS	High Performance Liquid Chromatography with Mass Spectrometry
HRE	Hormone-responsive elements
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
MR	Mineralocorticoid receptors
mRNA	Messenger RNA
PVN	Paraventricular nuclei
r	Correlation coefficient
RIA	Radio Immune Assay
SCC	Somatic cell count

1. HAIR: A TOOL TO EVALUATE THE HPA AXIS ACTIVITY

1.1 The hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis consists of a complex set of interactions between the hypothalamus, pituitary gland and adrenal cortex, which mediates the stress response through the actions of glucocorticoids (GCs). GCs are primarily involved in the metabolic homeostasis and are able to pass also the blood brain barrier. They regulate many body processes, including the cardiovascular tone, the body fluid volume, the immune system and inflammatory processes, the metabolism, some neurobiological processes and the reproduction system. A composite system such this is able to produce energetic metabolites and the resulting energy supply is used by the defence mechanisms to cope with the stressor. In contrast, excessive or inadequate basal activity or responsiveness of the HPA axis might impair development, growth and body composition and lead to a number of behavioural and acute or chronic somatic pathological conditions (Reul and de Kloet, 1985; Mormède et al., 2007; Chrousos, 2009).

Cortisol, the primary hormone of this axis, is an appropriate biological endpoint in the investigation of the HPA axis function in cattle, sheep, pig, mink, fox and fish (while corticosterone in birds and laboratory rodents). The cascade of events (**Figure 1**) that produce changes in cortisol release by the adrenals begins with the release of the corticotropin-releasing hormone (CRH) and the arginin vasopressin (AVP) by cells in the paraventricular nuclei (PVN) of the hypothalamus. CRH and AVP are released through capillary vessels to the anterior pituitary where they stimulate the release of adenocorticotropic hormone (ACTH) into circulation. ACTH is produced from cleavage of the precursor molecule proopiomelanocortin in anterior pituitary corticotrope cells. ACTH stimulates the biosynthesis and release of cortisol from the adrenal cortex (Mormède et al., 2007; Chrousos, 2009). Moreover, CRH acts also directly via receptors expressed throughout the brain having a role in regulating several neuroendocrine functions including behavior, food intake, reproduction, growth, immune function, and autonomic function (Sutton et al., 1982; Majzoub, 2006). Urocortin has been described as another CRF receptor ligand that plays important roles in the behavioral responses to stressors, being involved in stress-related physiology and in the control of the HPA axis (Ryabinin et al., 2012; Fox and Lowry, 2013).

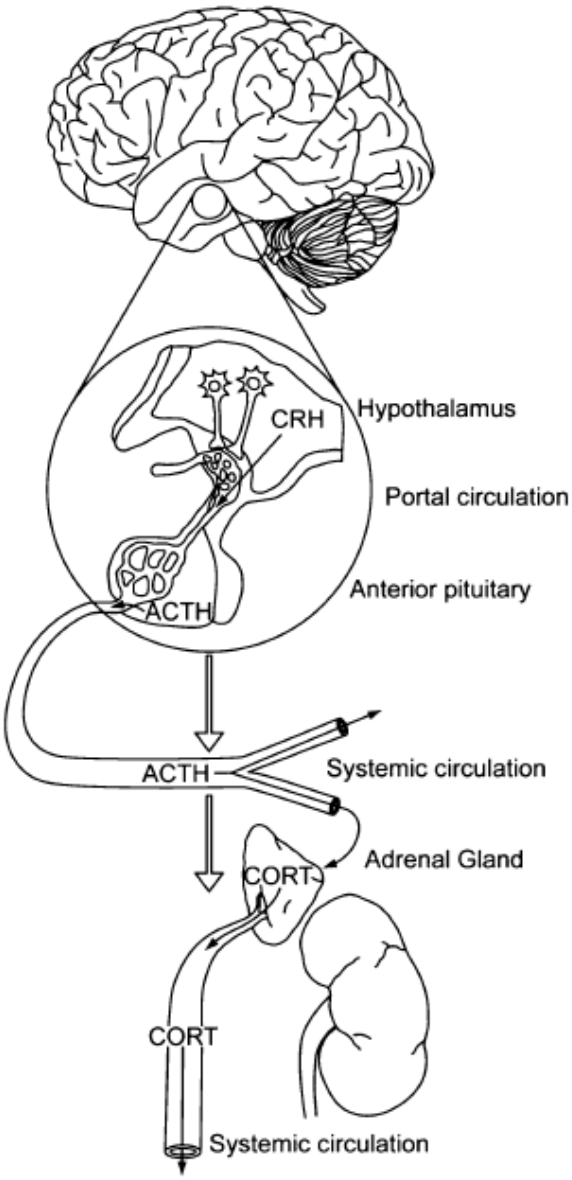

Fig.1. The HPA axis regulates the endocrine stress response with activation mediated by CRH-containing neurons in the hypothalamic PVN. The release of CRH onto cells of the anterior pituitary induces the secretion of ACTH into systemic circulation. At the adrenal cortex, ACTH stimulates synthesis and release of GCs (cortisol in humans and corticosterone in rodents). GCs then activate MRs and GRs providing a feedback signal to regulate HPA axis activity (Myers et al., 2012).

Approximately 95% of cortisol/corticosterone in the circulation is bound to a carrier protein (corticosteroid-binding globulin, CBG). In principle, only the free steroid has ready access to target cells. However, in some cases (e.g. in inflamed tissues) local serine proteases facilitate delivery by liberating free steroid from its binding globulin, while in others (e.g. pituitary gland) locally expressed CBG may limit access by absorbing free steroid (Buckingham, 2006).

It seems that the most important factor regulating the access of endogenous GCs to their receptors (mineralocorticoid or glucocorticoid receptors) is local metabolism of the steroids within the target cells by 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes, a phenomenon sometimes termed pre-receptor metabolism. 11β-HSD catalyses the interconversion of cortisol and its inactive metabolite cortisone (Buckingham, 2006). Two distinct isoforms of 11β-HSD have been cloned and characterised, type 1 and type 2 (11β-HSD1 and 11β-HSD2 (Edwards et al., 1996). The type 1 acts reactivating cortisone while the type 2 inactivates cortisol to cortisone. As 11β-HSD1 is found mainly in tissues in which the high affinity mineralocorticoid receptors (MR) are scarce but the low-affinity glucocorticoid receptors (GRs) are abundant, it has been argued that principal role of 11β-HSD1 is to amplify the local concentration of active GCs in those tissues in which the steroids have a key regulatory role, for example, the liver. There is also a growing interest in the role of 11β-HSD1 in the brain, particularly in the hypothalamus, hippocampus, cortex and cerebellum where the enzyme is expressed in abundance (Buckingham, 2006).

GCs are able to bind intracellular high-affinity MR (type I) or lower-affinity GRs (type II), which function as ligand-gated transcription factors to positively or negatively regulate gene expression by direct interaction with specific glucocorticoid response elements (GREs) in the promoter region. Access of the ligand-receptor complex to the GREs is effected via a process involving dissociation of the chaperone proteins and sequential phosphorylation, dimerisation and nuclear translocation of the receptor where will bind to specific DNA elements, the hormone-responsive elements (HRE) in promoter regions, affecting thus the transcription rate of corticosteroid-responsive genes (de Kloet et al., 1998). The biological actions of the steroids are thus generally slow in onset and persist for some time after the steroid has been cleared from the circulation. The affinity of the MR causes them to be extensively bound at low circulating levels of

GCs, and they are thought to be important in ambient glucocorticoid signalling processes (e.g., controlling basal secretion across the circadian cycle) (Dallman et al., 1989). In conditions where GCs concentrations rise markedly such as after stress, the glucocorticoid selective GR is activated, together with MR, which modulates appropriate stress responses by controlling and terminating stress-induced HPA reactions through the feedback regulation.

In addition to the classic genomic effects, GCs can also mediate rapid cellular responses through non-specific interaction with cellular membranes. It has been shown that at high concentrations GCs dissolve into membranes and can affect physicochemical membrane properties, as well as the activities of membrane-associated proteins (Buttgereit and Scheffold, 2002).

Non-genomic effects may also be mediated by the binding of GCs to the GR located in the cytoplasm or on the membrane (Evanson et al., 2010; Tasker and Herman, 2011). A role for the intracellular receptors is further supported by evidence that the activated GR complex may regulate post-transcriptional events including mRNA transcript stability, mRNA translation and post-translational processing. Such actions maybe particularly relevant to steroid effects manifest before the full transcriptional effects of the genomic actions are apparent (Buckingham et al., 1996).

Genomic effects of GCs normally take place within hours or days in the nanomolar range, while non-genomic effects of GCs may occur within seconds or minutes at significantly higher concentrations.

Many of the effects of GCs that are beneficial for short-term survival can be counterproductive or even deleterious if prolonged. Therefore, the activation and inhibition of GC release is a temporally regulated process involving rapid neuronal activation and efficient inhibition. The inhibition occurs as a negative feedback loop whereby cortisol binds to receptors in the pituitary and hypothalamus as well as the hippocampus and prefrontal cortex reducing or turning off the HPA axis response. This feedback action of cortisol participates in the return of the HPA axis activity to basal levels after stimulation (Manteuffel, 2002). Fast, non-genomic feedback inhibition of the HPA axis is mediated at least in part by GC signalling in the PVN, acting by a cannabinoid-dependent mechanism to rapidly reduce both neural activity and GC release. Endocannabinoid release causes presynaptic inhibition of glutamate release,

which reduces the neural activity of parvocellular neurons (Di et al., 2003). Forebrain genomic GC signalling is also a key component of feedback regulation, mediated via the prelimbic division of the prefrontal cortex presumably by genomic mechanisms (Herman et al., 2003). GCs also act in the brainstem to attenuate neuropeptidergic excitatory input to the PVN via acceleration of mRNA degradation, providing a mechanism to attenuate future responses to stressors (Zhang et al., 2009). Thus, rather than having a single defined feedback switch, GCs work through multiple neurocircuits and signalling mechanisms to coordinate HPA axis activity to suit the overall needs of multiple body systems (Myers et al., 2012).

1.2 Traditional biological matrices providing information on the HPA axis activity

In prior research, scientists have frequently used plasma, salivary, urinary, milk and faecal samples to measure cortisol levels.

Plasma is the most widely used sample to assay the HPA axis activity by cortisol evaluation in human and in a wide range of animal species. More commonly, plasma/serum cortisol has been used to determine acute reactions of animals to short-term stressors using a pre-exposure vs. post-exposure sample comparison (e.g., social separation (Higley et al., 1992; Meyer et al., 1975). A single plasma/serum sample only represents the HPA axis response at a particular moment in time.

Plasma concentrations of cortisol are characterized by a large variation. Ultradian, diurnal, and eventually seasonal rhythms have been described in several species (Follenius et al., 1987; Thun et al., 1981; Fulkerson and Tang, 1979). Furthermore, as described in human feeding may alter the diurnal cortisol rhythm by a mediator of the rise in cortisol secretion from the adrenals that is not yet known (insulin is a candidate mediator of the rise in 11β-HSD1 activity after the meals) (Stimson et al., 2014). Although the meal-induced release of cortisol has been described in humans many years ago (Follenius et al., 1982), it has not been specifically investigated in most farm animal. Cortisol plasma concentrations are also sensitive to many environmental factors. Latter activate a reaction described by Selye (1936) that sets the central nervous system in action, which activates the vegetative nervous system and the endocrine system through the hypothalamus and the pituitary gland. In this way GCs increase simply by catching and handling the animal. This artefact can be avoided, as suggested by Mormède et al. (2007), by taking the blood sample before the adrenal cortex has been activated, within 2-3 min of catching the animal or in alternative, samples can be taken by means of an in-dwelling catheter. Even if by venal puncture, blood sampling can be performed only by the vet. After spinning, plasma samples should be frozen immediately and stored at -20°C until analysis. Samples that are allowed to defrost long before being processed may have a lower cortisol concentration than at the time of collection. Moreover, plasma samples should not be thawed and frozen several times (Mormède et al., 2007).

In recent years, **saliva** sampling has provided an important alternative to blood sampling, particularly in human studies (Hellhammer et al., 2009). If compared with

blood, the sampling can be non invasive but on the other hand fluctuations in cortisol concentrations described for blood can still influence salivary cortisol concentrations. In fact, the correlation between salivary and free serum cortisol is well supported in the human literature, both being biomarkers for short-term measurement and both able to represent the diurnal fluctuations (Levine et al., 2007; Tunn et al., 1992; Poll et al., 2007). In sheep, salivary and free serum cortisol levels were also closely correlated. From observations after ACTH administration, it was concluded that the concentration of cortisol in sheep saliva is 10% that of plasma with no delay between the rise of cortisol in plasma and that in saliva (Mormède et al., 2007).

Saliva sample in sheep, goats and cattle, as described so far, can be obtained by aspiration from the mouth with a plastic suction tube (Fell et al., 1985; Fell and Shutt, 1986). This is described as not possible in young kids, probably because of a low rate of saliva production causing a damage by the suction tube, which results in bleeding and contaminations of the saliva sample with blood (Greenwood and Shutt, 1992). In pigs has been described an saliva sampling by allowing the animals to chew on swab for about 1 min until thoroughly moistened. The swabs should be then stored in test tubes and centrifuged before analysis (Parrott et al., 1989; Geverink et al., 1998). A special attention needs to be paid on the material from which swabs are made because can be an influence of them on the assay. Kidd et al. (2009) reported that the use of cotton or polyester swabs as the salivary collection method may over- or underestimate salivary cortisol concentrations. As showed by Poll et al. (2007), a reliable method to collect saliva samples can be the Salivette® swabs, since they are easier to handle and to process. In both the above described methods, sampling has to be done before the activation of the adrenal cortex can influence the blood cortisol level and consequently the saliva cortisol concentration. The saliva sampling can be performed by non-vet personnel. Moreover, as described before for plasma, all these kinds of collected specimens should be frozen immediately after sampling and stored at -20°C until analysis.

Urine is the main elimination route of cortisol. Urinary cortisol and its metabolites provide an integrated measure of their production over a period of time long several hours, thereby adjusting for the fluctuations in plasma levels. On the other hand the diurnal cycle found in urine cortisol secretion is similar to that in plasma samples. Urine

samples can be collected noninvasively by spontaneous urination (De Clercq et al., 2013; Capolongo et al., 2013). Otherwise, they can be collected by continuous aspiration from a urinal strapped to each animal (Berman et al., 1980) or by animal fitted with an inflatable vesical catheter that allow continuous urine collection (Hay et al., 2000), techniques that are more complicated to carry out on the field.

Cortisol can also be detected in excreted **milk** (Tucker and Schwalm, 1977). A clear disadvantage is that milk can be sampled only in lactating mammals as e.g. cows, goats, and ewes.

In measuring cortisol metabolites in **faeces** has to be considered that there are important differences between species in the proportion of cortisol metabolites excreted in the faeces (28% in sheep, 41% in ponies and only 7% in pigs), there are differences in the interval between the release of cortisol and its excretion via faeces (longer in pigs than in ruminants and ponies) and there are differences in the main metabolites excreted in faeces (in ruminants, but not in pigs and horses, the 11- and 17-dioxoandrostanes are the main excretory products) (Möstl et al., 1999; Palme, 2005; Möstl and Palme, 2002).

Faeces can be collected by a non-invasive method after defecation and directly from the bedding as suggested by Fureix et al. (2013) but because steroids are often not evenly distributed within faecal samples, homogenization of samples is recommended before their processing (Palme, 2005). In samples storage and processing is important to consider that the concentration of the 11- and 17-dioxoandrostanes increases in faeces at room temperature and even after freezing some of the 11- and 17-dioxoandrostanes producing enzymes are active, whereas enzyme activity is lost after heating the sample to 95°C (Palme et al., 2000). As saliva, urine and milk, faeces sampling can be performed by non-vet personnel.

Each of these sampling matrices has advantages and disadvantages, and these must be considered in determining which sampling matrix is best for answering the research questions at hand.

The recently developed technique of measuring cortisol from **hair** provides a powerful new tool with which to assess chronic HPA axis activity.

1.3 Hair

Hair cortisol is a novel marker of long-term cortisol elevation free from many of the methodological difficulties associated with plasma, salivary, urinary, milk and faecal cortisol (Dowlati et al., 2010; Steudte et al., 2011b). Segmental hair analysis has been successfully used in forensic, toxicological and doping research (Villain et al., 2004).

In the last decade has been shown that endogenously produced cortisol can be reliably measured in hair (van Uum et al., 2008; Kirschbaum et al., 2009; Koper et al., 2011). The use of hair provides the opportunity to measure long-term cortisol levels reflecting mean levels of the past months in an easy way without limitations caused by the pulsatility and circadian rhythm of cortisol or acute circumstances. Moreover, hair sampling is characterized by non-invasive collection and sampling can be performed at any time of the day, reducing the logistic impact. Sampling is simple and can be executed by a non-professionist (D'Anna-Hernandez et al., 2011; Dettenborn et al., 2012b, Gow et al., 2010).

1.4 Incorporation of lipophilic substances in hair

Knowledge is still limited about the exact mechanism whereby cortisol is incorporated into the hair shaft but because steroid hormones are lipophilic substances it can be relied on discovers made by forensic scientists for lipophilic drugs.

The major route of steroids incorporation in hair is suggested to be from the vascular supply by passive diffusion from blood capillaries into the follicular cells that generate the hair shaft (Barroso et al., 2011; Pragst and Balikova, 2006). Diffusion from tissues surrounding the follicle is likely to be an additional pathway by which cortisol eventually reaches the hair as suggested by Meyer and Novak (2012). Once the growing shaft emerges from the scalp, it is coated with sebum originating from the associated sebaceous gland along with sweat secreted by nearby eccrine glands (**Figure 2**).

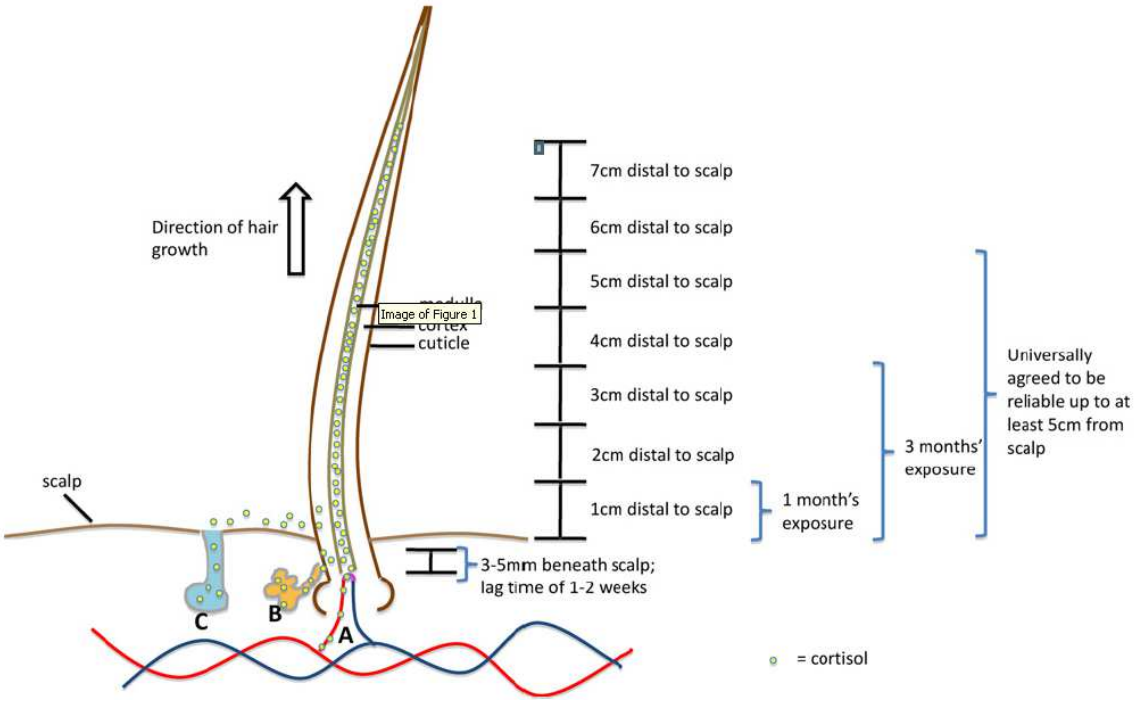

Fig.2. Proposed mechanisms for incorporation of cortisol into hair via blood (A), sebum (B), and sweat (C) (Russell et al., 2012).

In humans (Ito et al., 2005; Slominski, 2005; Slominski et al., 2007) and in guinea pigs (Keckeis et al., 2012) a cortisol synthesis in the skin (melanocytes, keratinocytes) is described - therefore, it remains an open question if the cortisol concentration in hair

reflects the cortisol concentration in the peripheral circulation or the local production (or both).

1.5 Comparison between hair and traditional biological matrices

In the early studies on the hair cortisol it was important to provide evidence of construct validity. In accord with Stalder and Kirschbaum (2012), the most direct validation of hair cortisol is to correlate data accumulated from repeated assessments of cortisol in hair, saliva, plasma and urine. Among healthy Chinese graduate students, a significant correlation (r=0.38) was found between hair cortisol analyzed in the most proximal centimeter (representing the most recent month) and average salivary morning cortisol derived from three weekly measurement days over four weeks (Xie et al., 2011). Hair cortisol and diurnal salivary cortisol (three samples per day over three days) were also correlated in pregnant women (r=0.24-0.57) (D'Anna-Hernandez et al., 2011). Steudte et al. (2011a) revealed a non-significant correlation (r=0.27) between hair cortisol in the first hair segment and diurnal salivary cortisol in participants with generalized anxiety disorder (six samples per day over two days). Finally, Sauvé et al. (2007) found significant correlations between hair cortisol and 24h-urinary cortisol (r=0.33) but not with plasma (r=0.06) or morning salivary cortisol obtained at a single time point (r=0.31).

Results from the aforementioned studies regarding validation of cortisol levels in hair through comparison with other cortisol measures are not compelling, since the correlation data are quite variable. Nevertheless, as plasmatic, salivary, urinary, faecal and hair cortisol assessments reflect different time frames (current state, one day, months respectively), strong associations are not expected.

1.6 Methods used to analyze cortisol in hair

Following the steroids extraction, methods to quantify hair cortisol include Radio Immune Assay (RIA), Enzyme Immune Assay (EIA), Enzyme Linked ImmunSsorbent Assay (ELISA), ChemiLuminescent Immuno Assay (CLIA) and High Performance Liquid Chromatography with Mass Spectrometry (HPLC/MS) or Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS). Gow et al. (2010) describe mass spectrometry as the “golden standard” of hair analysis, but these methods are relatively expensive and researchers use seldom mass spectrometry techniques to quantify hair cortisol (Cirimele et al., 2000; Raul et al., 2004). So far, hair cortisol concentration were measured by LC-MS/MS only in dogs (Bryan et al., 2013).

RIA method based on binding of ^{3}H-steroid by competitive adsorption is diffusely used for hair cortisol quantification (Accorsi et al., 2008; González-de-la-Vara et al., 2011; Corradini et al., 2013; Galuppi et al., 2013; Siniscalchi et al., 2013) as well commercial EIA/ELISA kits (Davenport et al., 2006, 2008; Martin and Réale, 2008; Dettmer et al., 2009; Bennett and Hayssen, 2010; Macbeth et al., 2010; Hamel et al., 2011; Fairbanks et al., 2011; Laudenslager et al., 2011; Bechshøft et al., 2011; Laudenslager et al., 2012; Malcolm et al., 2013; Ouschan et al., 2013; Terwissen et al., 2013). RIA and ELISA methods are sensible and less expensive than mass spectrometry techniques. Moreover, the coefficient of variation are generally below 10% (Kramer et al., 2009; Sauvé et al., 2007; van Uum et al., 2008), which is similar to the coefficients found with HPLC/MS (Raul et al., 2004).

It is important that the assay have a specificity for cortisol and a degree of sensitivity appropriate for the size of the samples being analyzed. Hair cortisol concentrations are generally reported in pg/mg.

1.7 Hair cortisol analysis in studies on the human HPA axis activity

For many years, forensic scientists have used hair samples to detect banned substances including stimulant drugs and anabolic steroids (Barroso et al., 2011). The first forensic papers to describe methods for detecting various corticosteroids (both natural and synthetic) were published in 2000 by Bévalot et al. (2000), Cirimele et al. (2000), and Gaillard et al. (2000), followed a few years later by quantification of endogenous cortisol and cortisone in the hair of normal male and female human subjects (Raul et al., 2004).

In just a few years since its inception as a new biomarker of the HPA axis activity, hair cortisol has been used in a wide variety of applications as chronic stress, endocrine disorders and neuropsychiatric disorders.

Human studies of hair cortisol and stress include individuals suffering from chronic pain (van Uum et al., 2008), individuals who were unemployed for at least 12 months compared with those who had jobs (Dettenborn et al., 2010), individuals consigned to shift work compared with day workers (Manenschijn et al., 2011b), endurance athletes who undergo severe physical stress (Skoluda et al., 2012), patients hospitalized with acute myocardial infarction compared with control patients (Pereg et al., 2011), alcohol-dependent individuals undergoing withdrawal compared with abstinent alcoholics or control subjects (Stalder et al., 2010) and newborn infants who required hospitalization in the neonatal intensive care unit compared with healthy newborns (Yamada et al., 2007).

Among the clinical applications of hair cortisol there is the determination of long-term changes in cortisol levels in patients with Cushing's syndrome or adrenal insufficiency. Initial studies showed that hair cortisol can play a useful role in monitoring disease progression and treatment efficacy in such patients (Thomson et al., 2010; Gow et al., 2011; Manenschijn et al., 2011a).

Recent work by Dettenborn and colleagues (2012a) found increased cortisol concentrations in hair from depressed patients compared with matched healthy controls. Luo et al. (2012) examined female adolescents who experienced a major earthquake in China in 2008. Subjects exposed to the earthquake had higher hair cortisol levels than controls who were not affected by the earthquake.

These studies illustrate that hair cortisol can provide an important addition to the existing literature on salivary, plasma or urinary cortisol concentrations in patients with different health disorders.

1.8 Applications of hair cortisol evaluation in the veterinary field

In addition to the broad use in humans even in the animal field there is a continuous increase in the application of hair matrix use for the study of the HPA axis activity. This axis regulates many biological processes such as energy balance, reproduction or immune responses, and is also activated by stress conditions. In domestic farm animals as in other mammalian species, certain environmental or management conditions have the potential to evoke the activation of the HPA axis that has an adaptive and homeostatic function (Minton, 1994).

1.8.1 Hair cortisol as a marker of the HPA axis activity in cattle

Given the importance of cortisol in monitoring the HPA axis activity, a first approach has involved the study on the distribution of hair cortisol concentrations in 229 healthy lactating Italian Friesian dairy cows from a single herd (Comin et al., 2012a; Peric et al., 2012c). In a range of values from 0.8 to 20.41 pg/mg, 50% of the evaluated animals showed hair cortisol concentrations lower than 3.13 pg/mg. Hair cortisol levels recorded are shown in the box plot (**Figure 3**).

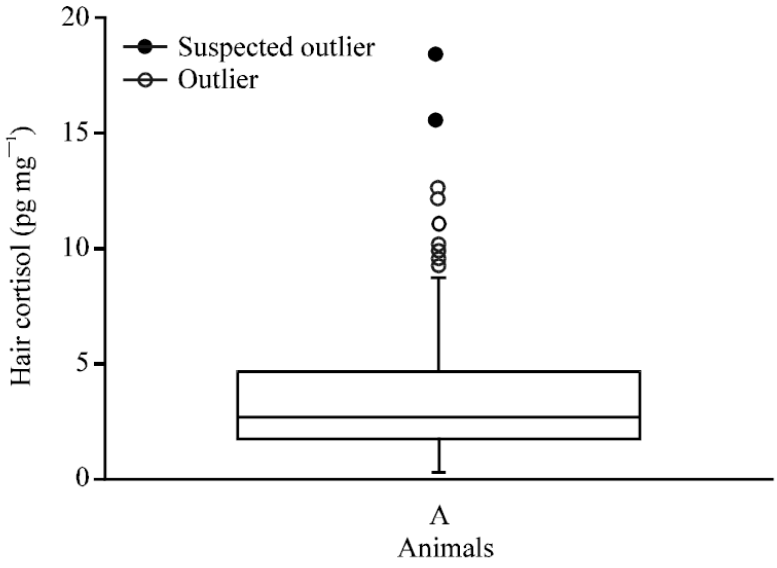

Fig.3. Hair cortisol levels measured in 229 multiparous lactating Italian Friesian dairy cows. Plots represent the median (horizontal lines), 25th and 75th quartiles (boxes) and the minimum and maximum cortisol values (whiskers) (Comin et al., 2012a).

The cows were classified into fourteen classes according to their hair cortisol levels and percentage frequencies for each class were calculated as showed in **Figure 4**. The 33% of the animals were concentrated in the hair cortisol class 2-2.99 pg/mg.

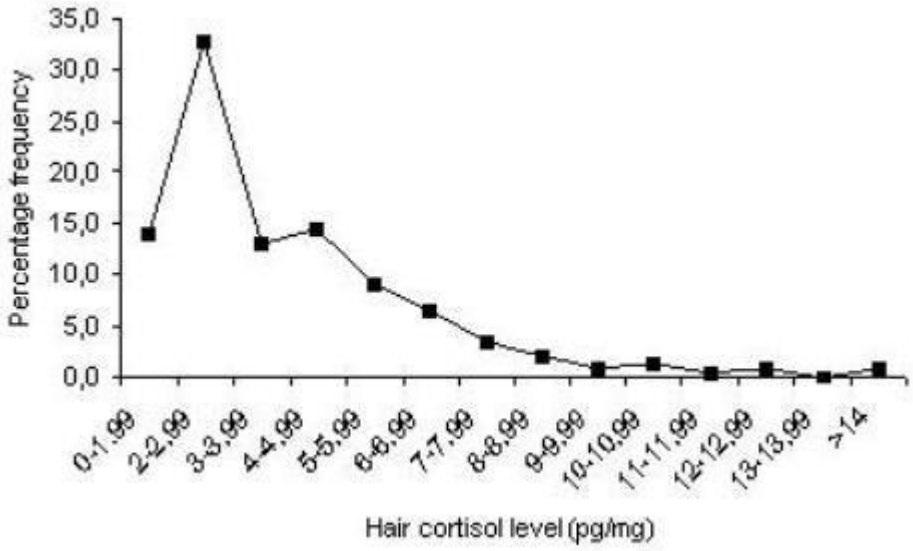

Fig.4. Percentage Frequency Curve according to hair cortisol classes (Comin et al., 2012a).

Considering that hair cortisol concentrations provide long term information, these levels are likely to be representative of the physiological range of variation in hair cortisol produced in healthy lactating dairy cows. The presence in the clinically healthy cows both animals with strongly activation HPA axis and animals with a low level of activity suggest that the perception of wellness in animals is often too anthropocentric. There is a dramatic difference in the sensorial sensitivity between dairy cows and humans (Fay and Popper, 1994; Fulwider et al., 2008; Jacobs, 1993).

Moya et al. (2013) found similar concentrations in the hair of beef cattle while González-de-la-Vara et al. (2011) found remarkably different in 15-day-old heifers and 2-year-old cows (114.5±14.43 and 12.15±1.85 pg/mg, respectively). This variability may be due to several factors such as age. In fact, neonatal calves always have higher blood levels of cortisol, also found in an analysis on the hair (Comin et al., 2008), as this hormone is essential for early delivery and neonatal development and maturation of several organ systems (Owen et al., 2005). Within the same species we must not underestimate other variables such as nutrition, season, hair color.

This preliminary study that describes the distribution of hair cortisol levels in healthy Friesian dairy cows was the starting point for monitoring the activity of the HPA axis in bovine at different physiological and environmental conditions.

A change in environmental conditions, such as the movement of 83 dairy cows from indoor winter farming to summer grazing pastures, has led to a significant increase ($P<0.01$) in hair cortisol concentrations (**Table 1**).

Trait	Farm			Period			SEM	*P*-value		
	F1	F2	F3	P1	P2	P3		F	P	F×P
Cortisol, (pg/mg hair)	2.2	2.7	2.7	2.1^{B}	2.9^{A}	2.6^{A}	0.10	ns	**	ns
FCM, (kg)	12.5^{AB}	13.6^{A}	11.2^{B}	14.9^{A}	12.4^{B}	10.0^{C}	0.38	**	**	**
SCS, (units)	2.91	3.47	3.31	2.60^{B}	3.36^{A}	3.73^{A}	0.224	ns	**	ns
BCS [1], (score)	1 [0–1]	1 [0–2]	1 [0–2]	1 [0–2]^{a}	1 [0–2]^{a}	1 [0–2]^{b}	–	ns	*	–

SEM: standard error of the mean.
**^{A,B,C}Within the same row and factor with unlike letters differ significantly at $P<0.01$.
*^{a,b}Within the same row and factor with unlike letters differ significantly at $P<0.05$.
ns: not significant.
[1] Welfare Quality Consortium score: 0 = too thin; 1 = normal; and 2 = too fat.

Tab.1. Estimated marginal means of hair cortisol level, fat corrected milk (FCM), and somatic cell count (SCC) and medians [min–max] of body condition score (BCS) recorded 7 (P1), 40 (P2) and 70 (P3) days after the start of grazing in three different farms (F1, F2, and F3) (Comin et al., 2011).

The increase in hair cortisol concentrations may be the consequence of the change from winter housing to high mountain conditions modifying the HPA response. Indeed, transfer of the animals involved a substantial change in breeding conditions, diet (from dry to fresh forage), as well as climate and altitude-related hypoxia that could all affect the nutritional status and metabolism of cows (Leiber et al., 2006). However, the limited increase in cortisol (all the mean hair cortisol concentrations recorded were comprised in the range described previously by Comin et al. (2012a) for healthy lactating dairy cows) indicates that the transition from winter housing to summer grazing did not activate so markedly the HPA axis in the animals. It is likely that on the farms the transition feed used, the low stocking density and the concentrate offered to animals during summer grazing helped the cows adapt to the mountain pasture conditions. In addition, the greater space of the pastures may reduce the stress of social mixing or hierarchy in dairy cows (EFSA, 2009). Only partially consistent with these findings, Kreuzer et al. (1998) found in 24 multiparous cows that had calved two to four weeks before a high altitude sojourn and fed only on pasture grass, a significantly higher

plasma cortisol after two months of alpine sojourn than initial lowland levels. Despite this, these authors suggested that metabolic adaptation to high mountain conditions requires about three weeks. The hair cortisol concentration enabled to monitor the trend shown by this hormone over two months of summer grazing, avoiding interactions due to the sampling procedure or circadian rhythm.

Significant increases (P<0.01) in hair cortisol concentrations were also recorded in cows clinically compromised that had recently suffered a disease (e.g. metritis, laminitis, mastitis) or physiologically compromised that calved 1 month prior to hair sampling (Group A *n*=218) compared to clinically healthy cows (Group B *n*=257) (Comin et al., 2013).

It has been shown that HPA axis dysfunction negatively impacts on animal health (Chrousos, 2009). Optimal basal activity and responsiveness of this system is essential for the well-being. By contrast, excessive or inadequate basal activity and responsiveness of the HPA system might impair development, growth and body composition, and lead to a host of behavioural and somatic pathological conditions.

In our study significantly lower levels were detected in Group B than Group A (P<0.01) suggesting different HPA axis activation between the groups (**Table 2**).

	Group A	Group B
Cortisol[a] (pg/mg)	5.12 [1.62–28.95][b]	3.29 [0.76–20.41][b]

[a] Difference according to the non-parametric Mann–Whitney *U*-test.
[b] Values in the same row with different superscripts vary significantly at $P < 0.01$.

Tab.2. Median [min-max] hair cortisol levels recorded in cows recently calving or suffering a disease (Group A; *n*=218) and in clinically healthy cows (Group B; *n*=257) (Comin et al., 2013).

Remarkably, the animals in Group B showed lower hair cortisol levels even when compared with each subgroup of animals that suffered a different pathologies disease or had recently calved (**Table 3**).

	Group							P-value[a]					
	Group B	INF	MAS	MET	PAR	LAM	MTO	Group B vs. INF	Group B vs. MAS	Group B vs. MET	Group B vs. PAR	Group B vs. LAM	Group B vs. MTO
Cortisol, pg/mg	3.29 [0.76–20.41]	6.40 [1.84–13.92]	4.07 [1.71–16.88]	5.96 [1.89–22.18]	5.15 [1.92–22.25]	5.23 [1.62–28.95]	6.35 [2.23–15.35]	< 0.01	0.042	< 0.01	< 0.01	< 0.01	< 0.01

[a] Difference according to the non-parametric Kruskal–Wallis *H*-test followed by the non-parametric Mann–Whitney *U*-test with post hoc Bonferroni correction.

Tab.3. Median [min–max] hair cortisol levels recorded in clinically healthy cows (Group B; *n*=257) and in cows recently calving or suffering a disease: inflammation (INF; *n*=20), mastitis (MAS; *n*=40), metritis (MET; *n*=22), parturition (PAR; *n*=78), lameness (LAM; *n*=38) or more than one condition (MTO; *n*=20) (Comin et al., 2013).

Increase in hair cortisol concentrations in animals clinically and physiologically compromised suggests that these cows were subjected to repeated HPA axis activation. Long-term changes in basal cortisol secretion could affect homoeostasis leading to an increased susceptibility to several diseases. Indeed cortisol is a pleiotropic hormone that affects all major homoeostatic systems of the body (Chrousos, 2009; Papadimitriou and Priftis, 2009) and under normal conditions, can provide a front line of defense against threats to homoeostasis (Mills et al., 1997).

The results obtained in this study were not always in agreement with those emerging from studies examining plasma cortisol levels. The data obtained with the hair approach are useful for detecting long-term activation of the HPA axis, and could be misleading if you wish to study activation of the axis in the short-term, such as in acute stress. Thus, the presented findings in cows with clinical mastitis were consistent with those described by Huszenicza et al. (2004), Kulcsár et al. (2005) and Lavon et al. (2008) who claimed that acute mastitis-induced secretion of inflammatory mediators increases the release of hypothalamic-corticotrophin-releasing hormone, which actives the adrenal axis. In the present study a significant correlation between the HPA axis activity and subclinical mastitis has been detected, contrary to reports by Forslund et al. (2010) and Lavon et al. (2010) that this disease did not affect plasma cortisol concentrations. According to Galvao et al. (2010) and Kulcsár et al. (2005), cows with metritis showed elevated plasma cortisol concentrations. In contrast, Forslund et al. (2010) detected no variations in plasma cortisol levels in dairy cows with mastitis or metritis. In this study an increase in hair cortisol concentrations in cows with laminitis

was also observed. These results are contradictory to those observed in studies by other authors (Ley et al., 1996; Walker et al., 2010), in which no differences in plasma cortisol levels were recorded between animals with or without laminitis. Almeida et al. (2008) detected serum cortisol levels approximately 49% higher than in healthy control cows but this difference was not statistically significant. Nevertheless, some authors have reported increased plasma cortisol concentrations in horses with laminitis (Ayala et al., 2012; Hodson et al., 1986). These differences among the different studies could be a consequence of the different biological samples used to measure cortisol levels. Hair reflects all hormonal variations day-to-day and provides a historical memory, unlike blood, which reflect cortisol concentrations at a single point in time.

The presence of clinically healthy animals with high concentrations of cortisol in these study could be explained by increased HPA axis reactivity. Moreover, mean hair cortisol concentrations recorded in this study were higher compared to the range described by Comin et al. (2012a) for healthy lactating dairy cows. High cortisol concentrations in clinically healthy animals could indicate a sub-clinical disease requiring a specific diagnostic method. In effect, hyper-activity of the HPA axis renders an organism more vulnerable to disease (McEwen, 2000). While it was initially thought that high cortisol was the result of diseases, there is mounting evidence that high cortisol actually plays a major role in inducing disease (Sapse, 1997).

The results revealed also individual differences in the extent of HPA axis activity in each animal. Individual variation in the HPA axis has been well documented in humans and attributed to genetic factors in twin and family studies (Linkowski et al., 1993; Inglis et al., 1999).

An individual trend in the hair cortisol concentrations has also been observed in Holstein Friesian heifers (*n*=142) compared with heifers Crossbreed F1 (*n*=148) (**Table 4**) (Peric et al., 2013a), pointing out that the response of an organism to maintain allostasis can be poorly predicted because of the many disturbing factors (genetic, environmental) (Golden et al., 2011). For instance, the bioavailability of corticosteroid hormones may result from genetic factors (Desautes et al., 2002; Remer et al., 2008), inherent adrenal sensitivity to adrenocorticotropic hormone, or levels of CBG (Gagliardi et al., 2010), 11β-HSD enzymes (Seckl, 2004), and glucocorticoid receptor levels (Nicolaides et al., 2010). Individual variation can also arise from environmental

influences in utero, during the early postnatal stage, or as a result of experiences later in life (Mormede et al., 2007; Scharf and Schmidt, 2012).

Item	Holstein-Friesian	Crossbreed
Heifers (no.)	142	148
Cortisol (pg/mg of hair)	5.38^{A} (1.91–27.95)	4.40^{B} (2.11–41.74)

^{A,B}Medians with unlike superscripts differ significantly at $P < 0.01$.

Tab.4. Medians (minimum–maximum) of hair cortisol concentrations of Holstein-Friesian and Crossbreed F1 Holstein heifers (Peric et al., 2013a).

As shown in **Table 4**, Crossbreed F1 heifers showed significantly lower (P<0.01) cortisol concentrations compared to pure Holstein-Friesian animals, pointing out a higher level of resilience and a better adaptability to the environment. Charney (2004) described that individuals with the highest levels of HPA axis activity will have the highest allostatic load. In contrast, individuals with lower, but not depressed, HPA axis activity will be characterized by a more resilient profile (Charney, 2004). Several researchers indicate that the welfare of purebred dairy cows has been compromised by high selection pressures that, in recent years, have increased the rate of inbreeding for most breeds. Crossbreed animals were found to be more economically efficient compared with the parental breeds (Maki-Tanila, 2007) and could be characterized by greater resilience to stressors. A study in young men examined the relationship between resilience (measured using the Resilience Scale for Adults) and HPA axis reactivity, pointing out that highly resilient individuals secrete less cortisol than less resilient individuals, but that phenomenon is not conductive to lower HPA axis reactivity (Mikolajczak et al., 2008). This characteristic could be very advantageous when the cow is in a biologically stressful state, such as during active milk production.

Hair cortisol levels in both Holstein-Friesian and Crossbreed F1 heifers were found to be higher than those observed in the previous studies (Comin et al., 2011; Comin et al., 2012a). Higher levels of cortisol in heifers may be due to several stressors, including weaning and the prepubertal and pubertal periods. It has also been observed that calves, similarly subjected to many stressors, have greater hair cortisol concentrations than cows (Comin et al., 2008; González-de-la-Vara et al., 2011).

Results described by this study helps us to better understand the differences in HPA activity and allostatic load between Holstein-Friesian and Crossbreed F1 heifers.

A breed influence has been also observed in a preliminary study designed to evaluate changes on hair cortisol concentrations of Simmental and Brown Swiss bulls relative to the beginning of semen production and the effects of hair cortisol concentrations on semen quality of bulls. Bulls contribute more to the overall reproductive success of the herd than any other individual animal, therefore, male sub-fertility has significant economic consequences. GCs inhibited reproductive functions in most domestic species studied (Calogero et al., 1999). Persistently high cortisol concentrations due to a situation of chronic stress can have detrimental effects on reproductive processes in animals. In bulls (Barth, 1993), dogs (Hatamoto et al., 2006), humans (Miesel et al., 1997) and rats (Retana-Márquez et al., 2003), stress induces a significant reduction in semen quality, especially due to a decrease in sperm concentration and motility, an increase in sperm defects (Calogero et al., 1999; Barth and Bowman, 1994; Tsantarlotou et al., 2002) and an impairment of antioxidant synthesis (Tsantarlotou et al., 2002; Hatamoto et al., 2006). The sperm cell is especially sensitive to oxidative stress, due to its relatively high content of polyunsaturated fatty acids in its plasma membrane that can be easily oxidized (Tsantarlotou et al., 2002; Hatamoto et al., 2006; Retana-Márquez et al., 2003). The blood cortisol levels has been used to investigate the role of the adrenocortical system in regard to the stress-mediated influence on the hypothalamic-pituitary-gonadal (HPG) axis.

All the animals included in this study were yearling bulls transferred from two Genetic Centers into artificial insemination center. The animals, before the start of semen production, were quarantined for 30 days as required by regulations of the national veterinary health surveillance service.

Hair cortisol levels in bulls (Peric et al., 2012b), before the relocation, were found to be higher than those observed in the previous studies in cows (Comin et al., 2011; Comin et al., 2012a). Higher levels of cortisol in bulls may be due to several stressors, including beginning of the pubertal period. The relocation produced in both breeds a significant increase ($P<0.05$) in hair cortisol levels (**Figure 5**) related probably to the change in environment and management to which the animals were subjected arriving at the artificial insemination center. A similar increase in hair cortisol levels was found

following relocation to a new environment in rhesus monkeys and in vervet monkeys (Fairbanks et al., 2011; Dettmer et al., 2012). In Brown Swiss bulls this increase was found to be two times higher than that recorded in Simmental bulls (unpublished data); it pointed out a lower level of resilience and a worse adaptability of Brown Swiss bulls to the new environment.

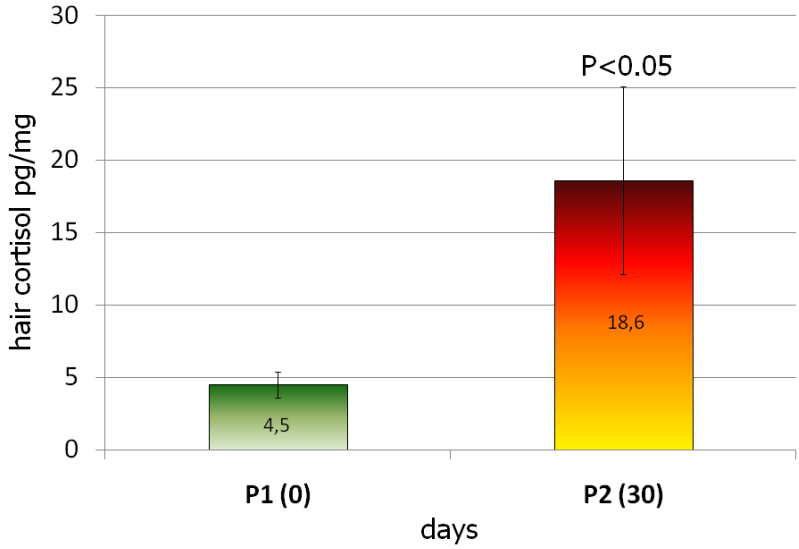

Fig.5. Mean ± SE hair cortisol concentrations in 12 Holstein Frisian bulls at the time of arrival (P1) at the artificial insemination centre and after 30 days (P2) (Peric et al., 2012b).

It was observed also that, one month after arrival at the artificial insemination center, bulls with hair cortisol concentrations >10.0 pg/mg produced ejaculates with a significant (P<0.05) lower spermatozoa concentration than bulls with hair cortisol concentrations <5 pg/mg (**Figure 6**) (Peric et al., 2012b); thus, producing even lower seminal doses.

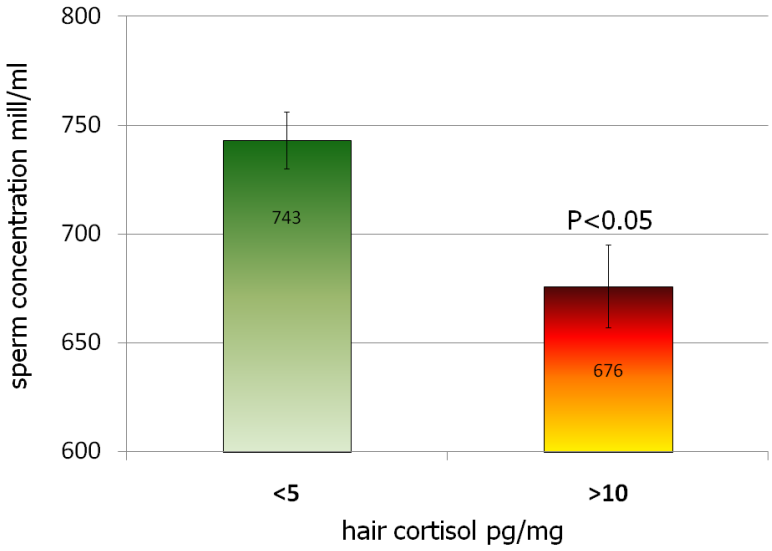

Fig.6. Mean ± SE spermatozoa concentration in 12 Holstein Frisian bulls in relation to different hair cortisol concentrations at 30 days after the arrival at the artificial insemination centre (P2) (Peric et al., 2012b).

These results indicate that hair cortisol monitoring could be a useful tool for the evaluation of HPA axis activity in relation to the beginning of semen production in bulls. Evaluate the effect of HPA activity on the HPG axis could be important not only for their potential economic implications but also for implications concerning animal welfare.

1.8.2 Hair cortisol and newborn

Due to its characteristics of reflecting extended periods of time (months to years) and for providing retrospective information, hair proved to be an excellent matrix also in the study of the activation of the HPA axis in newborn during the transition from the prenatal, the neonatal period and early months of life. Investigate hormones on hair provide a new interesting tool for cortisol noninvasive measurement in newborns useful for the study of both last intrauterine development fetal stage and for newborn adaptational monitoring. In this regard a few studies have been done in hair of foals.

Plasma cortisol concentrations in normal foals in the perinatal period are well known and are characterized by a further increase immediately after birth (>130 ng/ml) and subsequently decline 30 minutes after birth (20 ng/ml) (Silver et al., 1991). This fall in cortisol concentrations has also been studied in the hair matrix and a significant decline in foal hair cortisol concentrations from birth to 60 days of life was noted pointing

this matrix as useful in monitoring prenatal and neonatal HPA axis activity (Comin et al., 2012b). Possible explanations for this drop in the cortisol levels include 1) the progressive adaptation to extra-uterine life, 2) the end of the feto-maternal relationship that occurred through the placenta, and 3) the effect of environmental factors on the foal after birth. Many studies take into account the first two hypotheses (Silver et al., 1991; Rossdale et al., 1982; Panzani et al., 2009a; Panzani et al., 2009b; Veronesi et al., 2005; Troedsson and Sage, 2001; Cottril et al., 1991; Wilsher and Allen, 2003).

Since in horse foaling occurs from January to July in the northern hemisphere, was interesting to investigate temperature and lighting conditions because could affect the HPA axis and cortisol secretion in newborn foals during the process of neonatal adaptation (Montillo et al., 2012b; Montillo et al., 2014).

As reported in **Table 5**, the mean cortisol concentration of hair collected at 30 days of age was significantly (P<0.01) lower than that found at birth and none of the evaluated environmental factors (temperature, rainfall or day length) influenced the hair cortisol concentrations suggesting that rainfall, temperature and lighting conditions are not able to affect the production of cortisol in the long term. This is most likely because during pregnancy, the effects of all other external environmental factors are filtered through the mother, which considerably diminishes their impact. The cortisol that was find in this study in a foal's hair at birth is not produced by the fetal adrenal glands but is the result of the passage of maternal cortisol into the fetal blood stream through the placenta, as well as steroid production by the placenta.

	Gender		Sampling Time		SEM	Significance		
	Female	Male	ST1	ST2		G	ST	G×ST
Cortisol, (pg/mg hair)	47.63	47.91	60.37	35.17	0.065	ns	<0.01	ns

G: gender; ST: sampling time; G×ST: Gender × Sampling Time; ns: p> 0.05.

Tab.5. Estimated marginal means of hair cortisol level of foalings at birth (ST1) and at 30 days of age (ST2) (*n*=219) (Montillo et al., 2014).

Hair samples collected at 30 days of age reflect the cortisol accumulated between 15 days before foaling and 15 days of age, including the full peripartum period. Compared to the sample collected at birth, the cortisol measured at 30 days of age is the result of maternal and placental production, as well as production by the foal's

adrenal glands. The drop in levels by 30 days after birth indicates a significant reduction in the HPA axis activity that it is undoubtedly underestimated because the measurement includes the high concentrations of cortisol that occurred during birth. After birth, the effects of the climatic factors on the foal are not filtered through the mother; nevertheless, a chronic HPA axis activation in hair was not recorded. This study suggests that climatic factors such as temperature, rainfall and lighting during the perinatal period in foals has no significant effect on the hair cortisol concentrations measured at birth or 30 days of age. The absence of a significant effect from these factors suggests that compensatory mechanisms enable the foals to buffer environmental changes without a chronic variation in cortisol levels.

1.8.3 Hair and claws as matrices to provide a retrospective image of the HPA axis activity

Cortisol concentrations was detect also in hair and claws of newborn puppies and kittens (Meloni et al., 2012). Hair cortisol concentration has been previously studied in dogs and cats (Accorsi et al., 2008), but not in newborn puppies and kittens and not in claws. Several studies supported the use of human nails as target tissue for the measurement of HPA hormones (Warnock et al., 2010; Khelil et al., 2011) and for retrospective information such as hair samples.

Since cortisol plays an important role in fetal multi-organs final maturation, the study (Meloni et al., 2012) evaluated the reliability for hair and claws cortisol measurement in newborn puppies and kittens as a retrospective picture of final gestation cortisol accumulation. In canine and feline foetus body hair begins to grow at 45 days of gestation and claws are formed by 40 days of gestation, so samples taken at birth reflect cortisol accumulated during the last stage of pregnancy (Pretzer, 2008).

The results (**Table 6**) obtained from 32 born dead normal puppies and 8 born dead normal kittens showed that cortisol is higher in the hair of kittens compared to puppies. No significant differences were found between hair and claws cortisol evaluations within each species and also between canine and feline claws cortisol concentrations. As concerns at the comparisons between sexes neither hair nor claws cortisol levels differed in the two sexes in each species. On the basis of coat color,

statistics failed to show significant differences between cortisol concentration in fair and dark hair, in contrast to the results obtained in a previous study (Bennett et al., 2010).

Sample	Dog (n=32)	Cat (n=8)	Dog		Cat	
			Male (n=17)	Female (n=15)	Male (n=4)	Female (n=4)
Hair	64±27.16*	87±13.95*	71±27.90	57±25.17	92±10.68	82±16.26
Nails	66±55.41	76±62.20	59±58.05	73±53.19	49±28.37	103±79

*p<0.01

Tab.6. Cortisol concentrations (pg/mg) (mean ±SD) in born dead puppies and kittens hair and claws samples (Meloni et al., 2012).

This study provide a new interesting tool for cortisol noninvasive measurement in newborn puppies and kittens useful for the study of both last intrauterine development fetal stage and for newborn adaptational monitoring.

No one has ever measured the steroids in the claws of even-toed ungulates, and studies of this matrix are restricted to understanding the horn tissue's inner composition (Berker et al., 2007) and the biomechanical unbalancing of bovine claws (Raven, 1999).

A study (Peric et al., 2013b; Comin et al., in reviewing) carried out on 32 calves showed that the cortisol concentration in the horny shoe in calves from 0 to 30 days of age was significantly higher (P<0.05) than at 31 to 60 and 61 to 120 days of age (**Figure 7**).

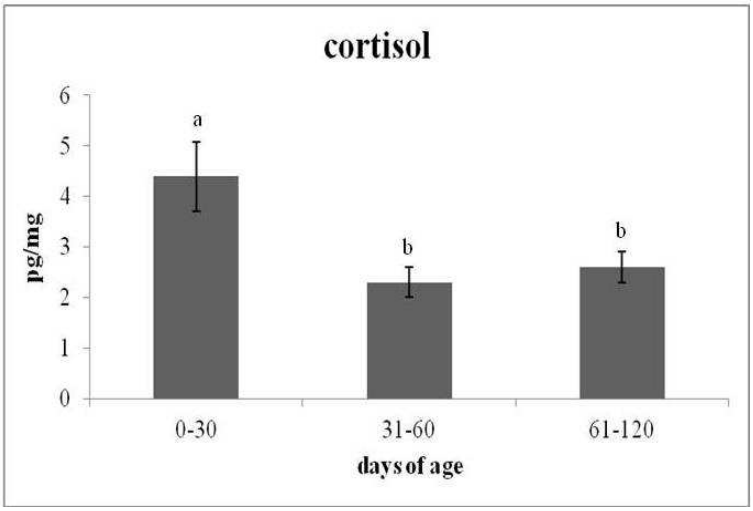

Fig.7. Horny shoe cortisol concentration in calves at 0-30, 31-60 and 61-120 days of age. The data are expressed as the mean value ± se. [a,b] indicates a statistically significant difference (P<0.05) (Comin et al., in reviewing).

Calves are a category of animal that is still free from any podal disorder due to excessive weight load, dietary, environmental or production disequilibrium.

The horn samples from the calves seem to suggest that the soles of the claws of calves behaved like human nail and hair samples; they demonstrated, in fact, the same memory effect (McMillen et al., 1995; Gow et al., 2010; Fourie et al., 2011). The cortisol concentrations in the calves' horn samples follow the same trend as samples of hair from calves and foals during peripartum (Comin et al., 2008; Comin et al., 2012a). The decrease in cortisol concentration in the calves' horn samples was in agreement with results obtained from Comin et al. (2008) in studies examining hair cortisol levels of calves at parturition and at least 6 months old. A similar trend of decreasing cortisol levels was detected in foal hair samples from birth to 90 days of age (Montillo et al., 2012a; Comin et al., 2012b).

This memory effect was not found in cow's claw (Comin et al., in reviewing). It is important to consider that even-toed ungulates use their claws to walk and overload them with body weight. This aspect is less significant in calves than in the milking adult cow.

Several studies suggest that hormones play critical roles in the normal development of the claw horn and correct keratin formation (Tomlinson et al., 2004). In particular, Hendry et al. (1999) found that glucocorticoids impact the maturation of keratinocytes through regulation of protein synthesis: hydrocortisone inhibited keratin protein synthesis in bovine hoof-tissue explants (Hendry et al., 1999). Epidemiologists have yet to identify a causative relationship between systemic glucocorticoid concentration and laminitis in dairy cows, resulting from production of an inferior claw horn (Goff and Horst, 1997). Instead, progesterone, a steroid hormone elevated in pregnancy, has been shown to have anti-inflammatory and immunosuppressive properties and to increase keratinocyte proliferation (Urano et al., 1995).

An experiment performed on 24 pregnant milking Friesian cows, clinically healthy and lacking any claw disorders, studied the claw matrix in dairy cattle by measurement of two steroid hormones, progesterone and cortisol, which are involved in the formation and growth of the horn. The samples were taken from the fore claws because they are

more stable than the hind claws, so they generally show fewer foot diseases and remain healthier (Raven, 1999).

In the cow claws, the progesterone concentrations of the different sole areas were not significantly different. However, an animal effect was observed (P<0.01) (**Table 7**). The horn progesterone concentrations recorded in the dairy cows ranged from 14.32 pg/mg to 103.66 pg/mg. For each individual animal, variability was found between the areas belonging to the same claw. Therefore, the horizontal variability is independent of the sampling area but is subject to the animal's individual reaction.

	Horizontal area					P-value	
	A	B	C	D	SEM[1]	Area	Animal
P4 pg/mg	36.9	39.77	28.07	33.33	2.213	ns[2]	< 0.01

[1]SEM = standard error of the mean.
[2]ns = not significant.

Tab.7. Progesterone concentrations of varied sole areas (Comin et al., in reviewing).

The results recorded in **Table 8** show significant differences between the progesterone concentrations of the transverse sections of the sole (P<0.01). Moreover, a significant effect of the animal was also observed (P<0.01). The progesterone levels significantly decrease from the outside to the inside of the sole.

	Vertical area										P-value	
	S0	S1	S2	S3	S4	S5	S6	S7	S8	SEM[1]	Area	Animal
P4 pg/mg	52.44[a]	42.01[ab]	42.9[ab]	30.04[bc]	30.46[bc]	22.31[c]	21.87[c]	19.75[c]	17.34[c]	1.338	< 0.01	< 0.01

[a-c]The superscript letters indicate statistically significant differences for the area's effect.
[1]SEM = standard error of the mean.

Tab.8. Progesterone concentrations at the sole's transverse section (Comin et al., in reviewing).

The cow claws, lacking any foot disorders, despite similar progesterone concentration between the horizontal areas, showed a high individual variability. This means the incorporation of progesterone in the different areas of the same claw was inhomogeneous, which could be due to a different response to mechanical stimulation of the hoof. Variability was also demonstrated by analysis of the transverse sections,

where the progesterone decreases from the distal to proximal sections. These data were unexpected because progesterone plasma levels are high and constant at pregnancy (Bradford et al., 1972; Mukasa-Mugerwa et al., 1989; Astiti et al., 2013). This unexpected variability of progesterone levels could be because dairy cows, like all even-toed ungulates (Artiodactyla), use their claws to walk, and overloaded areas of the claw present higher growth (Brizzi, 2008). This specific pressure, caused by an uneven load, produces an increased blood supply that could induce a higher steroid incorporation.

The present research shows how mature cow claws function as an active and bidirectional matrix because they constantly interact with the inner and external environment. So, unlike hair and human nail but similar to skin, the claws of mature dairy cows cannot be studied as a matrix to provide a retrospective image of HPA axis activity.

1.8.4 Hair cortisol and relocation

The hair was also an excellent matrix for the study of the HPA axis activity as a result of the relocation. The transfer of animals, their relocation and the impact of new staff dedicated to their care are factors capable of generating stress in any animal, particularly in rabbits, which are very sensitive to external stimuli and easily frightened (Cabezas et al., 2007). In accordance with this, a study showed an increased hair cortisol concentration related to an environmental change (transfer at the animal facility) (Peric et al., 2012a; Peric et al., in reviewing-a) and to the change of personnel at the facility (Peric et al., 2012a; Peric et al., in reviewing-b).

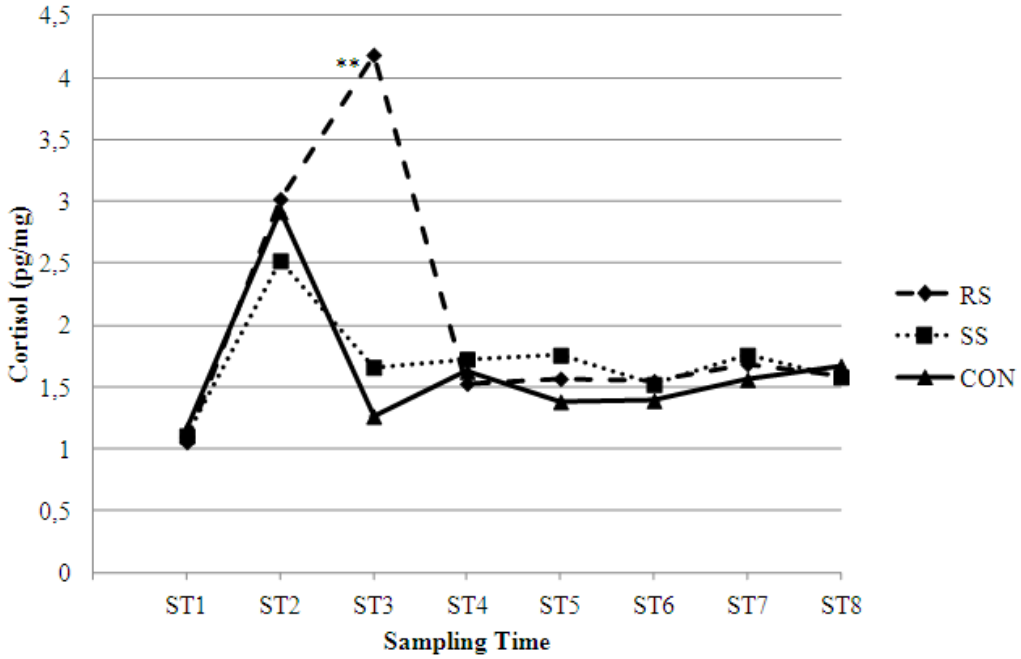

Fig.8. Changes in hair cortisol level in rabbits undergoing real surgery (RS *n*=19) or sham surgery (SS *n*=19) and in the control (CON *n*=19) group, recorded 0 (ST1), 40 (ST2), 80 (ST3), 120 (ST4), 160 (ST5), 200 (ST6), 240 (ST7) and 280 (ST8) days after the beginning of the trial. Rabbits were subjected to surgery on the 40th day after the beginning of the trial. Data are presented as the means ± SEM. ** indicates a statistically significant difference (P<0.01) between RS and CON group. To assess the effect of surgery and sampling time, repeated measure ANOVA analysis was used from ST3 to ST8 (Peric et al., in reviewing-a).

From the results obtained (**Figure 8**) it is evident that, in spite of the protocol and the rules related to the animal welfare that have been strictly followed, the transition from the rabbitry to the animal breeding facility led to an increased activity of the HPA axis over a long period of time, likely due to the particular sensitivity of rabbits to stress. A similar increase in hair cortisol concentrations was found in rhesus monkeys (Davenport et al., 2006, 2008) and in vervet monkeys (Fairbanks et al., 2011, Dettmer et al., 2012) following their relocation to a new environment. Interestingly, as showed in **Figure 8**, a surgery was performed at a time when the cortisol concentrations were three times higher than those recorded at the beginning of the trial.

Moreover, as showed in **Figure 9** a similar increase in hair cortisol concentrations to that induced by the change in the environment (transfer at the animal facility) was observed after a programmed change in the qualified staff at the facility (day 280). All the other parameters (e.g., nutrition, environment) were kept constant.

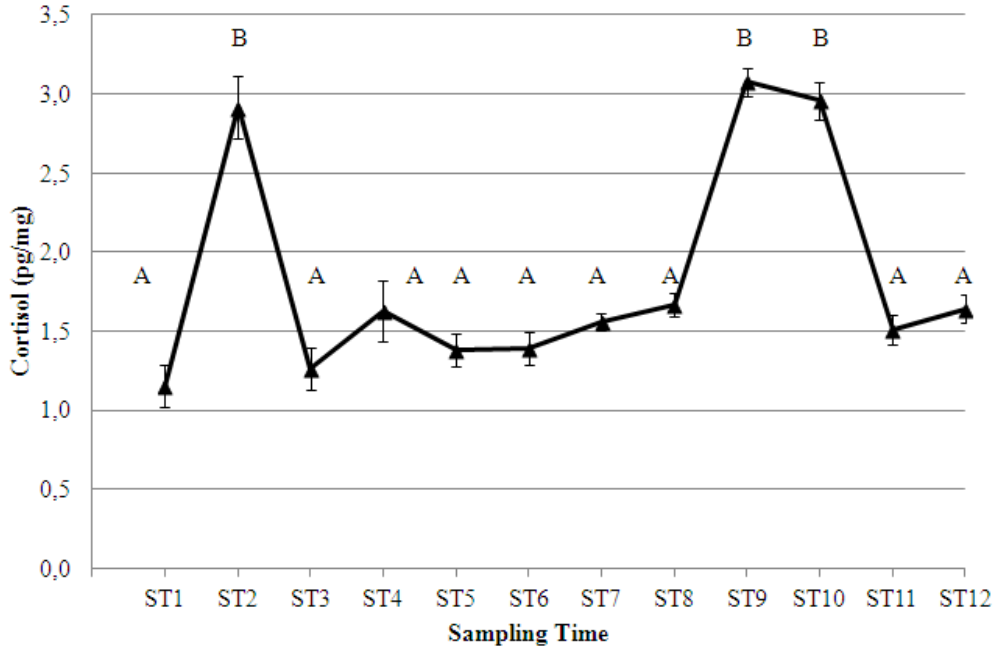

Fig.9. Hair cortisol level recorded 0 (ST1), 40 (ST2) , 80 (ST3), 120 (ST4), 160 (ST5), 200 (ST6), 240 (ST7), 280 (ST8), 320 (ST9), 360 (ST10), 400 (ST11), 440 (ST12) days after the beginning of the study in which at days 0 and at day 280 rabbits (*n*=19) were subjected to the change of enclosure and personnel respectively. Data are presented as means ± SEM. ^{A,B} indicates a statistically significant difference (P<0.01) (Peric et al., in reviewing-b).

The change of personnel at the facility required an activation of the HPA axis to cope with the new environmental situation, highlighting the fact that rabbits are easily frightened and need to be handled with great care (Brewer, 2006). Human-rabbit interactions are less likely to cause stress during husbandry or scientific procedures if staff behave in a way that is compatible with the natural behavior of the rabbit. As rabbits can recognize and discriminate between different humans (Davis and Gibson, 2000), positive contact with familiar humans in the form of handling, training and general habituation to human contact will provide interest for the animals and reduce stress when they are handled for procedures (Jezierski and Konecka, 1996).

In conclusion, environmental changes or a change in personnel can activate the HPA axis in rabbits. Consequently, a protocol for the conditioning and habituation of rabbits to humans can reduce the variation in experimental outcomes, thereby providing a more robust model (Joint Working Group on Veterinary Care et al., 2008; Verwer et al., 2009). Animals that are standardized as much as possible are important prerequisites for reproducible animal experiments (Nicklas et al., 2002). Moreover, measuring hair cortisol in rabbits could help the farmer or veterinarian monitor the well-

being of their animals. Hair cortisol level evaluation allows us to evaluate when the environmental change that occurred with the transfer from the rabbitry to the animal breeding facility no longer stimulates the HPA axis activity and to verify the achievement of the animals' equilibrium with the surrounding environment.

1.8.5 Hair analysis and mechanisms that require further investigation

Even though the literature on hair cortisol measurement is growing quickly, many other aspects of hair analysis still require a discussion, e.g., how the steroids are incorporated into the hair shaft and if the hair pigmentation can have an impact on the steroids concentration. It is also important to take the cortisol from the sebaceous glands and sweat glands that is deposited on the hair surface into account. Evaluate this aspect could provide new potentialities of the hair matrix. In a preliminary study cortisol measured in unwashed beard hair from 5 healthy adult men showed a comparable daily trend with salivary cortisol (**Figure 10**). Salivary and hair cortisol at 8:00h were significantly higher than cortisol level at 11:00h and 23:00h ($P<0.001$) (Esposito et al., 2012).

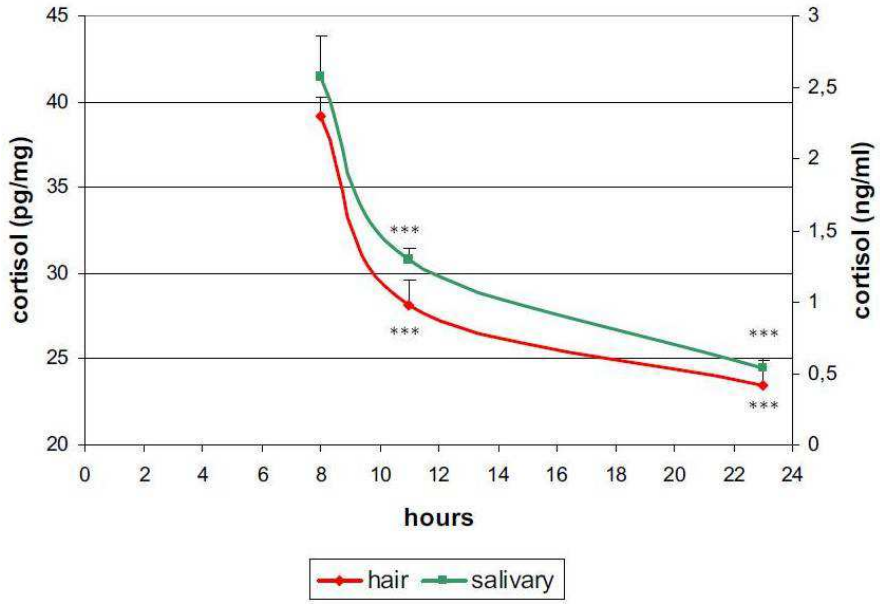

Fig.10. Saliva and hair cortisol levels (Esposito et al., 2012).

The comparable daily trend between hair and salivary cortisol may be due to external contamination of hair originating by the sebaceous glands and sweat glands because the hair had not been washed.

Another issue that deserves attention is the anatomical area of hair sampling since different growth rates and species specific depth of the hair follicle in various body sites have been reported. A study in 8 rabbits pointed out that rabbits kept in stable environmental conditions showed no significant differences in hair cortisol levels among the different body sites (**Figure 11**) (Comin et al., 2012c).

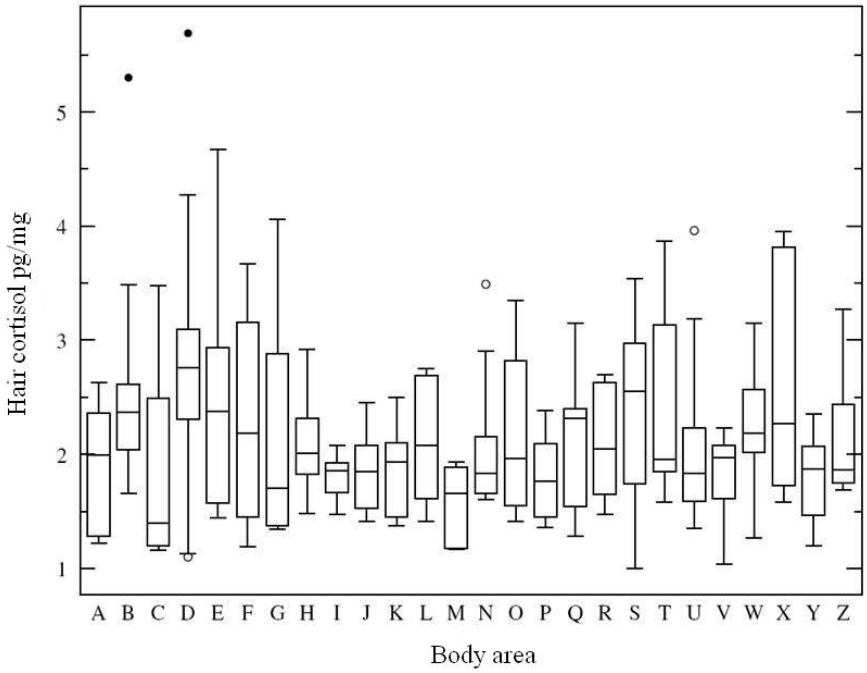

Fig.11. Hair cortisol levels determined at the 26 body sites in the eight rabbits. Plots represent the median (horizontal lines), 25th and 75th quartiles (boxes) and the minimum and maximum of cortisol values (whiskers). ° suspected outlier; • outlier (Comin et al., 2012c).

This means that throughout the study period, in the absence of stressful stimuli that could activate the HPA axis, hair showed similar cortisol concentrations regardless of length. In fact, at the time of sample collection, hair lengths were measured for each body region and the lengths of hairs collected from the different areas differed significantly ($P<0.001$). On the other hand, in situations characterized by uncontrolled environmental conditions it is important keep the hair sampling site constant across the subjects and to use the re-shaving technique to easily overcome the issue of different growth speed and different deepness of the follicle in the skin. At the same time will be established a known timeline of cortisol incorporation avoiding to obtain a mix of hair that has incorporated hormones over different time periods and will be collected also

always the same hair colour, overcoming in these way some of the questions on hair that are still opened.

2. CONCLUSIONS

Matrices such as plasma, saliva, urine, faeces whose characteristic is to provide punctual information, have allowed an extensive study on acute activation of the HPA axis. The study of a chronic activation of this axis has been hampered by the lack of a reliable matrix because none of the previous matrices provides a truly long-term index of the HPA axis activity.

Hair is a new matrix able to provide long-term retrospective measures of cumulative cortisol secretion. Sampling is characterized by non-invasive collection method, can be performed by a non-professionist and can be stored at room temperature.

Hair cortisol provides a new approach to monitor the long-term HPA axis activity (over periods of weeks to months) without limitations due to pulsatility and circadian rhythm of cortisol or acute environmental disturbances such as those linked to restraining the animal. Hair cortisol can be used as a retrospective calendar of the HPA axis activity and provides information on its chronic activation comparable to that supplied by glycated haemoglobin in the diagnosis of diabetes.

For decades hair analysis has been widely used and validated in human medicine to assess the HPA axis activity. Recently, the application has been started also in the veterinary field and it might be useful to measure easily and non-invasively the activity of the HPA axis in relation to chronic stressful situations and animal welfare. These aspects are particularly important in livestock because several environmental or management conditions experienced by domestic farm animals (e.g. crowding, mixing of unfamiliar animals, transportation, weaning, etc.) have the potential to activate the HPA axis.

In our study the setup of hair cortisol measurement in cattle has allowed to:

- identify in healthy Friesian dairy cows:
  a) a physiological range (0.8-20.41 pg/mg) of variation in hair cortisol concentrations and that the 50% of the animals had levels below 3.13 pg/mg;
  b) data non-normally distributed, pointing out an individual behaviour in the activation of HPA axis and showing that the response of an organism to

maintain allostasis can be poorly predicted because of the many disturbing factors (genetic, environmental);

- highlight such as environmental, managerial and physiological conditions like:
  a) the movement of dairy cows from indoor winter farming to summer grazing pastures,
  b) the transfer of yearling bulls from Genetic Centers into artificial insemination center,
  c) when dairy cows are clinically compromised because they had recently suffered a disease or are physiologically compromised

  can modify the activity of the HPA axis and increase significantly cortisol levels;
- point out a breed influence on the HPA axis activation:
  a) between Holstein Friesian and Crossbreed F1 heifers which showed cortisol concentrations significantly lower and thus were characterized by a more resilient profile that allow a better adaptability of crossbreed animals to the environment and highlights the importance of crossbreed traits for profitability in dairy farming. This result support the hypothesis that crossbreed animals respond better to environmental changes and stressors and are more economically efficient compared with the parental breeds;
  b) between yearling Simmental and Brown Swiss bulls which were transferred from Genetic Centers into artificial insemination center.

From these studies emerges that hair cortisol measurement in cattle can be considered as a valid indicator in the evaluation of the HPA axis chronic activation and can be an useful tool for vets and farmers in the assessment of the animal welfare. Indeed, a chronic activation of the HPA axis in dairy cattle might prevent them to fully express the genetic potential and to maximize the reproductive efficiency. Our findings indicate that hair cortisol could serve to identify among clinically healthy dairy cows the individuals with the highest levels of HPA axis activity and the highest allostatic load. These subjects are less resilient, less adaptable to changes in environment and management and they are likely to experience health and reproductive difficulties given the known connection between elevated activity of the HPA axis and infertility.

So, hair cortisol measurement could be candidate as a useful approach to identify animals that have a high activity of the HPA axis and thus a high allostatic load. As seen on a sample of 475 multiparous cows, this condition is correlated with a higher incidence of altered physiological status between animals. Obtaining in time information about the HPA axis activity may allow the implementation of strategies to increase the resilience of the dairy cows that are close to the delivery and beginning a new lactation. This could improve the productive and reproductive efficiency of the animal.

Due to its characteristics of reflecting extended periods of time (months to years) and for providing retrospective information, hair proved to be an excellent matrix also in the study of the activation of the HPA axis in newborn during the transition from the prenatal, the neonatal period and early months of life. The activity of the HPA axis depends on many factors such as genetic heritage, placental-fetal interactions, maternal care, peripartum and prepubertal period that are able to program the brain to confer vulnerability or resilience and have a direct effect on the individual ability in responding to environmental changes. The activity of the fetal HPA axis is modulated by the environment that characterize the mother life during pregnancy and important mother stress suffered during this period can impact the functionality of the fetal HPA axis later in life.

In conclusion, hair analysis in research constitutes a highly promising method and in human medicine has already found many applications not only in the forensic but also in the clinical field. The study of cortisol in hair as a tool to evaluate the HPA axis activity could open new horizons in practical applications on the veterinary field.

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APPENDIX 1: PUBLICATIONS BY DR.SSA TANJA PERIC

PUBLISHED PAPERS:

a) peer-reviewed journals:

1) Comin A., Prandi A., **Peric T.**, Corazzin M., Dovier S., Bovolenta S. 2011. Hair cortisol levels in dairy cows from winter housing to summer highland grazing. *Livestock Science.* 138: 69-73.

2) Esposito P. L., Comin A., **Peric T.**, Montillo M., Mascolo M., Tubaro G. and Prandi A. 2012. Experimental indicators of ergonomic wellness and quality of life salivary and hair cortisol. *Work.* 41: 5442-5445.

3) Comin A., Zufferli V., **Peric T.**, Canavese F., Barbetta D., Prandi A. 2012. Hair cortisol levels determined at different body sites in the New Zealand White rabbit. *World Rabbit Science.* 20: 149-154.

4) Comin A., **Peric T.**, Montillo M., Faustini M., Zufferli V., Cappa A., Cornacchia G. and Prandi A. 2012. Hair cortisol levels to monitor hypothalamic-pituitary-adrenal axis activity in healthy dairy cows. *Journal of Animal and Veterinary Advances.* 11: 3623-3626.

5) Comin A., **Peric T.**, Corazzin M., Veronesi M.C., Meloni T., Zufferli V., Cornacchia G., Prandi A. 2013. Hair cortisol as a marker of hypothalamic-pituitary-adrenal axis activation in Friesian dairy cows clinically or physiologically compromised. *Livestock Science.* 152: 36-41.

6) Celiberti S., Gloria A., Contri A., Carluccio A., **Peric T.**, Melillo A., Robbe D. 2013. Sexual Hormone Fluctuation in Chinchillas. *Veterinary Clinics of North America: Exotic Animal Practice.* 16 (1): 197-209.

7) **Peric T.,** Comin A., Corazzin M., Montillo M., Cappa A., Campanile G., Prandi A. 2013. Hair cortisol levels in Holstein Friesian and Crossbreed F1 heifers. *Journal of Diary Science.* 96: 3023-3027.

8) Montillo M., Comin A., Corazzin M., **Peric T.**, Faustini M., Veronesi MC., Valentini S., Bustaffa M., and Prandi A. 2014. The effect of temperature, rainfall and light conditions on hair cortisol concentrations in newborn foals. *Journal of Equine Veterinary Science.* doi:10.1016/j.jevs.2014.01.011

b) not peer-reviewed journals:

9) Prandi A., Comin A., **Peric T.**, Montillo M., Omodeo S.G. 2010. Nuovo approccio non invasivo per la valutazione del benessere animale. *Quaderno SoZooAlp.* 6: 183-191.

PAPERS IN REVIEWING:

a) peer-reviewed journals:

10) **Peric T.**, Comin A., Corazzin M., Montillo M., Canavese F., Stebel M., Prandi A. Hair cortisol in New Zealand White rabbits subjected to spine surgery. *Laboratory Animals.*

11) **Peric T.**, Comin A., Corazzin M., Montillo M., Canavese F., Stebel M., Prandi A. Hair cortisol concentrations in New Zealand white rabbits in a facility environment. *The Journal of the American Association for Laboratory Animal Science (JAALAS).*

12) Comin A., **Peric T.**, Magrin L., Corazzin M., Cornacchia G., Prandi A. Study of hormonal steroid concentrations in the Italian Friesian's claw. *Journal of Dairy Science.*

13) Meloni T., Comin A., Rota A., **Peric T.**, Contri A., Veronesi MC. IGF-I and NEFA concentrations in fetal fluids of term pregnancy dogs. *Theriogenology.*

ORAL COMMUNICATIONS:

a) national congresses:

14) **Peric T.**, Meloni T., Montillo M., Faustini M., Comin A., Pantaleo S., Prandi A. 2012. Individual hair cortisol as a tool for monitoring HPA activity in healthy dairy cows. *Proceedings of 10th Congress of Italian Society of Animal Reproduction (SIRA 2012) 1st Balkan Conference on Biology and Reproduction in Farm Animals and in Aquaculture, Tirana, Albania, 23-25 May, 2012.* Società Italiana di Riproduzione Animale, Castellana Grotte, Italia. ISBN: 978-88-907328-0-5. 147-149.

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