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VALIDATION OF A NEW METHOD TO ASSESS THE LONG-TERM HYPOTHALAMIC-PITUITARY-ADRENAL ACTIVITY USING HAIR GLUCOCORTICOIDS AS BIOMARKERS.

- Studies on pigs and laboratory Sprague-Dawley rats

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ABSTRACT

Validation of a new method to assess the long-term Hypothalamic-Pituitary-Adrenal Activity using hair glucocorticoids as biomarkers. - Studies on pigs and laboratory Sprague-Dawley Rats

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The evaluation of chronic activity of the hypothalamic-pituitary-adrenal (HPA) axis is critical for determining the impact of chronic stressful situations. The potential use of hair glucocorticoids as a non-invasive, retrospective, biomarker of long term HPA activity is of great interest, and it is gaining acceptance in humans and animals. However, there are still no studies in literature examining hair cortisol concentration in pigs and corticosterone concentration in laboratory rodents. Therefore, we developed and validated, for the first time, a method for measuring hair glucocorticoids concentration in commercial sows and in Sprague-Dawley rats. Our preliminary data demonstrated: 1) a validated and specific washing protocol and extraction assay method with a good sensitivity in both species; 2) the effect of the reproductive phase, housing conditions and seasonality on hair cortisol concentration in sows; 3) similar hair corticosterone concentration in male and female rats; 4) elevated hair corticosterone concentration in response to chronic stress manipulations and chronic ACTH administration, demonstrating that hair provides a good direct index of HPA activity over long periods than other indirect parameters, such adrenal or thymus weight. From these results we believe that this new non-invasive tool needs to be applied to better characterize the overall impact in livestock animals and in laboratory rodents of chronic stressful situations that negatively affect animals welfare. Nevertheless, further studies are needed to improve this methodology and maybe to develop animal models for chronic stress of high interest and translational value in human medicine.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic Hormone
AI	Artificial Insemination
ANOVA	Analysis of Variance
AP	Anterior Pituitary
AUC	Area Under the Curve
BD	Before Delivery
CORT	Cortisol and Corticosterone
CRF or CRH	Corticotropin-Releasing Factor (Hormone)
CUS	Chronic Unpredictable Stress
CV	Coefficient of Variation
EIA	Enzyme Immunoassays
ELISA	Enzyme-Linked Immunosorbent Assays
GC	Gas Chromatography
GR	Glucocorticoid Receptors
HPLC	High-Performance Liquid Chromatography
IMOch	Chronic Immobilization
MCR	Mineralocorticoids Receptors
ME	Median Eminence
MS	Mass Spectrometry

PD	Pregnancy Diagnosis
PVN	Paraventricular Nucleus of the hypothalamus
RIA	Radioimmuno-Assay
RT	Room Temperature
SC	Subcutaneous
SD	Standard Deviation
SEM	Standard Error of the Mean
SNK	Student-Newman-Keuls
SPSS	Statistical Program for Social Sciences
VP	Vasopressin
WT	Weaning Time

1. INTRODUCTION

The recognition of chronic stress and the deleterious impact can have on human health and on animal welfare is critical and still not completely understood. This is mostly due to a lack of agreed definition of stress, as well as the absence of objective measures. Moreover in animals, the veterinarian can evaluates animal's condition just considering its appearance and behavior that are specific for each specie. For these reasons, to control and to reduce the effects of stress and to ameliorate our animals well-being, some guidelines have been introduced.

For example, in livestock animals, welfare measures has been taken (EFSA, 1997) in order to standardize breeding procedures. However, animal stressors are linked to many subjective and environmental parameters. In fact, commercial pigs face several problems during their rearing and stressors can seriously affect their welfare turning into: diseases, decreased reproductive performance and economic loss for the farmer. In the past years, attention has been focused mostly to acute stress while, in intensive breeding, the most adverse stressors are chronic. In fact, the main chronic stressors (i.e.: temperature, photoperiod, seasonality, housing condition) are known to affect hormonal mechanisms (i.e.: swine summer infertility) but it is still unclear how they act.

In laboratory animals science, manipulations and environmental factors (i.e.: cage size, noises, odors, cleaning and the presence of humans and other animals) are part of the stimuli presented to an animal facility every day, influencing animals behavior and physiology and contributing to their welfare. Nowadays, standardized environmental conditions are used to reduce the variability within a particular study or between studies, thus facilitating the detection of treatment effects and increasing the reproducibility of results across laboratories. However, some uncontrolled environmental effects on the animals and theirs manipulation can be: a primary source of artefacts, they affects laboratory animal well-being and are often overlooked by ethical review committees. Previous studies suggest that routine procedures are intrinsically stressful to laboratory animals, but they are based on physiologic parameters reflecting acute stress (i.e.: plasma levels of ACTH, corticosterone, prolactin or glucose, heart rate, blood pressure and behavior) but not on the overall impact over long periods.

In physiology, it is widely accepted the idea that exposure to these systemic and emotional stressors involves a rapid activation of the hypothalamic pituitary-adrenocortical (HPA) axis, resulting in the stimulation of adrenocortical secretion of glucocorticoids. The primary glucocorticoid in rodents is corticosterone, while in humans, nonhuman primates and most mammals is cortisol. They regulate numerous biological processes and play a critical role in the proper adaptation of the organism to stressors (Sapolsky et

al., 2000). One particularly relevant function of glucocorticoids is to inhibit the activation of the HPA axis caused by stressors through negative feedback-loops exerted at different levels, including the pituitary gland, the hypothalamus and some extra-hypothalamic areas, such as the hippocampal formation and the medial prefrontal cortex (Armario 2006). If exposure to the stressor is prolonged and the stress response continues, this can lead to a syndrome of distress and to deleterious effects on health (McEwen 1998).

Finally, the activation of the HPA axis is considered a gold standard biomarker of acute stress (Armario et al. 2012), but the ability of glucocorticoids to reflect long term HPA axis activity is limited. Thus, establishing biomarkers of chronic stress is a great challenge.

The potential use of hair glucocorticoids as a non-invasive, retrospective, biomarker of long term HPA activity is of great interest, and it is gaining acceptance in humans and animals. In fact, to date, glucocorticoid concentrations have been analyzed mostly in blood serum, saliva, urine and feces, and each of these approaches has clear limitations.

Glucocorticoid levels in blood and saliva reflect acute changes in the HPA axis, show high intra-individual variation and are strongly affected by environmental disturbances (Hellhammer et al., 2007) and circadian and ultradian rhythms (Lightman et al., 2008). Currently, urine and fecal samples are widely accepted as a non-invasive technique and they reflect a longer period of

HPA activity: 24-48 hours. However, they require repeated sampling and are subjected to cross-contamination. More importantly, none of these matrices is able to reflect the long-term accumulative impact of chronic stress exposure.

Therefore, the potential use of hair as a non-invasive, retrospective biomarker of long term HPA activity is gaining acceptance because it has been recognized as a biomaterial that accumulates glucocorticoids over weeks to months and that could eventually reflect chronic stress (Davenport et al., 2006).

In literature, studies in humans have investigated the association between long-term stress exposure and hair cortisol levels, covering a wide range of physical and emotional stressors (Yamada et al., 2007, Van Uum et al., 2008, Dettenborn et al., 2010; Karlén et al. 2011; Grassi-Oliveira et al., 2012). Contrarily, only two papers have examined the chronic hair cortisol response to chronic stress in laboratory rhesus macaques and vervets monkeys (Davenport et al., 2006; Fairbanks et al., 2011). Additionally, to our knowledge, there is no report on the technical validation of the measurement of glucocorticoids in the hair of commercial sows neither in laboratory rodents as Sprague-Dawley rats.

Thus, the aims of the present work were: a) To develop and validate a non-invasive method for measuring hair cortisol concentration in sows and corticosterone concentration in rats, b) To demonstrate that hair matrix in breeding sows is useful to analyze long-term changes of cortisol in standard

breeding condition. c) to provide evidence that supports the hypothesis that hair glucocorticoid is representative of the free rather than the total fraction of circulating glucocorticoids, d) to demonstrate that hair corticosterone in Sprague-Dawley rats reflects long term HPA axis activity.

2. REVIEW OF THE LITERATURE

2.1 Concepts of Stress

Stress was originally defined by Selye in 1950 as:

"*The non-specific response of the body to any noxious stimulus*".

Later, Vigas (1984) refined this concept defining stress as:

"*A state of emergence created in the organism either by exteroceptive and interoceptive stimuli that profoundly alter homeostasis in a way that cannot be solved by specific homeostatic physiological mechanisms, or by stimuli that have no direct impact on homeostasis but have a reasonably probability to be followed by a real challenge to homeostasis*".

Besides, Chrousos (2009) described the stressor as a stimulus that threatens homeostasis and the stress response is the reaction of the organism aimed to regain it. The term "*homeostasis*" was coined for the first time by Cannon (1932). He supposed that many physiological variables have a preferred set-point and that a deviation of it is counteracted by physiological responses which are aimed at restoring the optimal level. However, to date, we can consider this definition almost meaningless and several papers (Levine and Ursin, 1991; McEwen, 1998; Day, 2005; Levine, 2005; Romero et al., 2009) underlined its ambiguity, supporting the idea that all activities of an organism directly or indirectly concern the defense of homeostasis.

Another problem is to define the term “*stressor*” and when it could be considered an important menace to homeostasis, to physical and psychological health. In fact, there are studies interpreting the presence of a stress response as an indicator of a stress exposure, independently from the definition of the stressor or the stress response (Armario, 2006). Otherwise, other researchers believe that stress should be considered as a process that includes the stimulus, the processing of this input and the behavioral and physiological output (Levine and Ursin, 1991; Levine, 2005).

Regarding the adaptive nature of the stress response, Selye (1976) introduced the term “*distress*” and “*eustress*” in order to figure out the differences between the maladaptive and the adaptive consequences of it. Anyhow, several authors are still debating and it still appears extremely difficult to clearly dissociate these two terms (McEwen and Wingfield, 2003; de Kloet et al., 2005; Korte et al., 2005; Dallman, 2007). Furthermore, it is suitable talking more about uncontrollability and/or unpredictability of the stressor (Koolhaas et al., 2011).

This conflicting information emphasize the importance and the complexity of the stress investigation that to date is not yet completely understood while being the center of the attention both in human and veterinary research.

2.2 The HPA axis and its activation

The hypothalamic-pituitary-adrenocortical axis (HPA) promotes an organisms' ability to cope with environmental stressors and facilitates behavioral adaptation (McEwen, 2003; de Kloet et al., 2005). Thus, exposure to systemic and emotional stressors are perceived by the limbic system involving a rapid activation of the HPA axis with the final stimulation of the adrenocortical secretion of glucocorticoids. Stress induced activation of the HPA axis is the result of stimulatory signals arriving at the paraventricular nucleus of the hypothalamus (PVN). In the PVN, there are parvocellular neurons that mainly synthesize corticotropin-releasing factor (hormone) (CRF or CRH) and other peptides as well (i.e.: vasopressin, VP) and send axonal projections to the pituitary portal blood of the external zone of the median eminence (ME). Upon activation, these neurons release CRH, VP and other secretagogues, which reach the corticotrope cells of the anterior pituitary (AP) to activate the synthesis and release of the adrenocorticotropic hormone (ACTH). Circulating levels of ACTH act on the zona fasciculata of the adrenal cortex to stimulate the synthesis and secretion of glucocorticoids.

In most mammals, including humans, the main glucocorticoid is cortisol, but there are also some levels of corticosterone. In rodents, the predominant glucocorticoid is corticosterone, and in the particular case of rats and mice they

have only corticosterone (Armario et al., 2012). Cortisol and corticosterone will be collectively termed CORT in this introduction.

Commonly in literature CORT is defined as "*the stress hormone*" because it is released in higher doses under stressful situations and travels through blood in both protein-bound (inactive CORT) and free (bioactive CORT) forms. This steroid hormone regulates numerous biological processes (i.e.: fat and glucose metabolism, blood pressure, inflammatory and immune responses) and it has a critical role in the proper adaptation of the organism to a stressor (Sapolsky et al., 2000; Staufenbiel et al., 2013). One of the main function of CORT is to inhibit the activation of the HPA axis caused by the stressor through negative feedback-loops exerted at different levels, including: the pituitary gland, the hypothalamus and some extra-hypothalamic areas such as the hippocampal formation and the medial prefrontal cortex (Armario, 2006). As described in literature, CORT operates in the context of the humoral and nervous signals that mediate the stress response and not alone (de Kloet et al., 2005; McEwen, 2007). CORT deadens the initial stress reactions to a given stressor (the defense), and prevents them from overshooting (Munck et al., 1984; Sapolsky et al., 2000). However, the main point is to understand how CORT activity can change from protective to harmful.

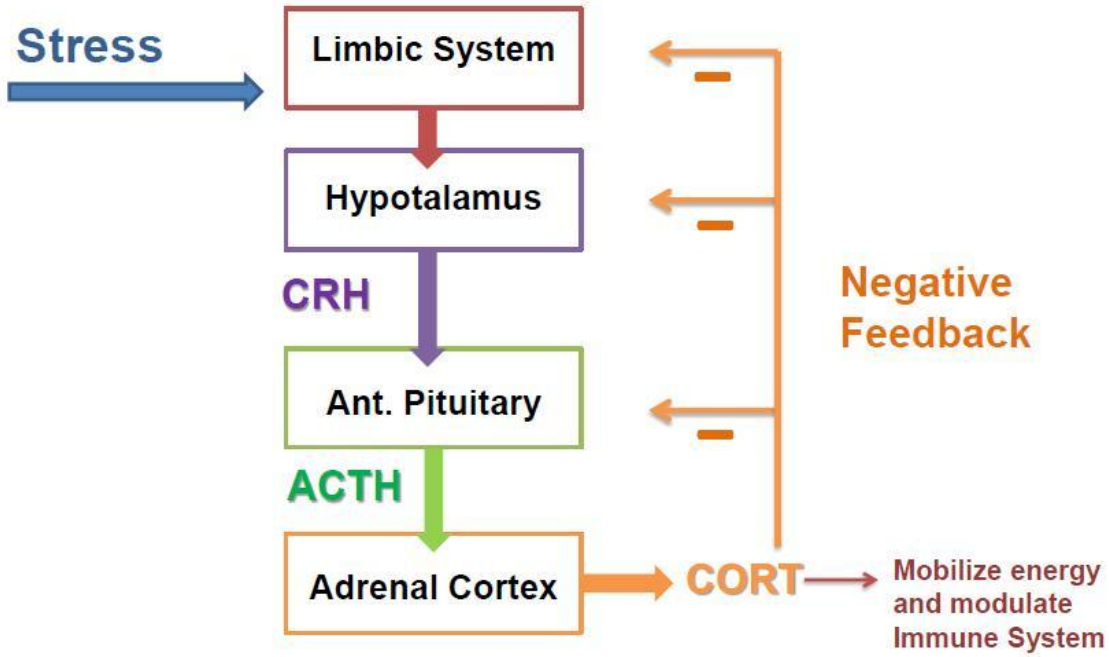

Figure 1. Hypothalamo-Pituitary-Adrenocortical Axis (HPA axis) activity:

- Stress is perceived by limbic system
- Neurons in the limbic system activate the HPA axis:
  - CRH neurons in hypothalamus release CRH at median eminence
  - CRH stimulates release of ACTH from cells in the anterior pituitary
  - ACTH stimulates both synthesis and release of glucocorticoids from adrenal cortex
  - Glucocorticoids act in part to mobilize energy to cope with the stressor
  - Glucocorticoids restrain the HPA axis by inhibiting hormone release at the level of the hypothalamus, pituitary, and higher brain regions (limbic system)

2.3 Definition of homeostasis, allostasis and allostatic load

In general, the source of the stress, the stressor, may be actual or perceived, and it is divided in two definitions: psychological and physiological (Selye, 1950). McEwen (1993) supposes that there are enormous individual differences in how people respond to a challenge situation and this could depend on two principal factors. The first is how the single individual perceives and interprets the situation. For example, if situations are perceived as a menace then behavior and physiological responses led to a negative consequence. The second aspect of individual differences concerns the good physical condition of the body. For this reason, metabolic imbalances, immunosuppression or diseases can increase the vulnerability of individual to stress (McEwen, 1993).

The stress response consists of three phases: 1) stress reaction, 2) recovery, 3) adaptation (Oitzl et al., 2010). These phases are considered extremely important for the processes underlying memory storage. In fact, strong emotions that are promoted by CORT are remembered best and consolidated through glucocorticoid receptors (GR) mediated actions. Otherwise, behavioral responses that are no more relevant are extinguished by CORT (Bohus and de Kloet, 1981; Oitzl and de Kloet, 1992) and the incoming information to the current situation is suppressed (Pu et al., 2007). These

actions are GR-dependent but they need the concomitant action of mineralocorticoids receptors (MCR) and of other stress signals as CRH, VP, and catecholamines. During the stress reaction phase, catecholamines (noradrenaline and adrenaline) are released with CORT and the speed and the magnitudes of them depend on the specific stressor (Pacáak and Palkovits, 2001). Noradrenaline or adrenaline work through the nervous system and within seconds, thereby enabling immediate physical reactions associated with the so called: "*flight-or-fight response*", known as the acute stress reaction (Staufenbiel et al., 2013). Although the physiological response to short-term stress is usually adaptive. Under normal circumstances the body attempts to maintain homeostasis in spite of the stressor and the increased release of glucocoticoids are terminated by their self, as previously described (**Figure 1**).

Physiological responses of the autonomic nervous system, HPA axis, metabolic, cardiovascular and immune systems lead to an adaptation of the organism to challenges. These processes, called "*allostasis*" by Sterling and Eyer (1998), are essential to keep homeostasis and the brain plays an important role in the regulation of them. However, it is important to understand that allostasis involves mechanisms that change the controlled physiological variable by predicting which level will be needed to meet anticipated demand (Koob and Le Moal, 2001). Thus, allostasis can be considered the physiological response as a response to a prediction. A better prediction of the demands shifts the integrated response. In this way, any mediators (i.e.: adrenal

hormones, cytokines, neurotransmitters etc.) act on tissues and organs receptors to produce a change that will be adaptive to behavior, metabolism, immune system and cardiovascular system in the short term (Koolhaas et al., 2011). The state of chronic deviation of this regulatory system from its normal operating level has been defined "*allostatic state*" by Koob and Le Moal (2001).

However, these allostatic systems could also cause problems for the body if they turn into overactive or underactive. The adaptation price that promotes pathophysiology has been defined as "*allostatic load*" by McEwen in 1998:

"*Allostatic load is the wear and tear on the body and brain resulting from chronic overactivity or inactivity of physiological systems that are normally involved in adaptation to environmental challenge.*"

He identified three types of physiological responses that make up allostatic load: type 1) frequent stress; type 2) failed shut-down and 3) inadequate response to challenge. In brief, he concludes that this imbalance arises for too much repeated stress, but it also can be the result of the adaptive systems that are out of balance and fail to shut-down or, alternatively, systems that fail to turn on adequately. Besides, allostatic load negatively affects the immune system and the brain function, especially the hippocampus, which is endowed with high levels of receptors for adrenal steroids.

In summary, the response to acute stress promotes allostasis or adaptation while long-term stress leads to deleterious consequences. These include the dysregulation of the HPA axis associated with the development of mental health disorders or hypertension, cardiovascular disease, obesity, chronic obstructive pulmonary disease or asthma, risk of ulcerative colitis, reduced fertility, poor pregnancy out come and exacerbation of skin conditions such as psoriasis.

2.4 Lack of markers for chronic stress

The activation of the HPA axis is considered as a gold standard biomarker of short-term stress (Armario et al., 2012) and several research groups have been focusing on the way in which sympathetic responses can be quantified during acute stress. Otherwise, the ability of glucocorticoids to reflect long-term HPA axis activity is still limited due to the nature of the traditional matrices in which CORT has been sampled. Investigators have been trying to validate methods for assessing CORT through blood, saliva, urine and feces in order to assess the long-term HPA axis activity and to better characterize chronic stress. However, each of these approaches has demonstrated clear limitations and high variability.

2.4.1 Blood and Saliva

CORT levels in blood and saliva reflect transient changes in the HPA axis, show high intra-individual variation and are strongly affected by environmental disturbances (Hellhammer et al., 2007) and circadian and ultradian rhythms (Lightman et al., 2008). Samples need particular storage requirements and the collection of them implies restraining the animal. In addition, the act of obtaining sample via venipuncture could be itself a source of stress (Vining et al., 1983). Measuring CORT in blood samples assess total CORT that includes both bound-protein (inactive fraction) and free CORT (bio active fraction). Consequently, total blood CORT is affected by changes in

levels of CORT-binding globulin that can result in increases in total CORT concentration measured, even though there is no increase in stress or free CORT concentrations (Russell et al., 2012). Contrarily, salivary CORT reflects bioactive fraction and its concentration is well correlated with blood concentration. Although saliva collection is considered "*less invasive*", it requires a lengthy training process (Davenport et al., 2006) and training could be not successful in all cases (Lutz et al., 2000). Furthermore, commercial saliva absorption materials can be flavored to make them more palatable, but this method yields inconsistent CORT results and sometimes citric acid, added to increase salivation, could be not well tolerated by each individual (Dreschel and Granger, 2009). Both blood and saliva samples provide a measurement of the CORT concentration at a single time point and can be used to test acute changes but they cannot reflect the long-term HPA axis activity.

2.4.2 Urine and Feces

CORT values obtained from urine and fecal samples reflect somewhat longer periods of HPA axis activity (from 12 h up to 24 h). They are not invasive techniques, not affected to acute variability of CORT concentration than blood and saliva and they assess just the bioactive and free CORT fraction. However, collection of such samples in laboratory animals might be difficult when subjects are housed in pairs or more, and problems could arise from cross-contamination of urine and feces (Davenport et al., 2006). Corticosteroids may

not be uniformly distributed within fecal samples and may be inversely related to fecal output (Hayssen et al., 2002), while urine samples can be difficult to collect, resulting in incomplete data sets (Stephen and Ledger, 2006). Excretion patterns are variable and the collection to assess long term activity could be intensive for technicians and need particular storage conditions.

In conclusion, all of these techniques described, induce measurement error and there are several confounding variables that could negatively influence the comparability of existing studies in this scientific area (Staufenbiel et al., 2013). More importantly, none of these matrices is able to reflect the long-term accumulative impact of chronic stress exposure and the HPA axis activity over longer periods of time.

2.5 Hair: a biomarker of long term HPA activity

Over the last 25 years, hair matrix gained increasing attention and recognition for the retrospective investigation of chronic drug or anabolic steroids abuse in humans. Hair differs from other materials used in forensic studies (i.e.: such as blood, saliva, feces and urine), because of its solid and durable nature that provide a long-term memory enabling retrospective investigation of chronic substance abuse. The first elements analyzed in hair to document history exposure were toxic metal ions (i.e: Tl, As, Pb and Hg) (Chatt and Katz, 1988; Kijewski, 1993). In 1979, Baugmartner et al. were the first to extract hair of heroin users and determined opiate content by radioimmuno-assay (RIA). In 1980, Klug performed the first chromatographic detection of opiates in hair previously disintegrated with NaOH. This study demonstrated how the concentration along the hair shaft differed and corresponded with the time course of drug intake. Afterwards, in 2000 it has been published the first forensic papers describing methods for detecting various natural and synthetic corticosteroids (i.e.: Bévalot et al., Cirimele et al. and Gaillard et al., 2000) followed a few years later by quantification of endogenous CORT and cortisone in the hair of healthy male and female (Raul et al., 2004). Since then, many researchers investigated this new technique and its numerous advantages as non-invasiveness and the possibility to use this matrix as a retrospective biomarker of CORT exposure. However, CORT hair analysis does not have validated procedures, needs relevant guidelines throughout the testing process

and there are many outstanding questions and gaps in our knowledge that need to be addressed.

2.5.1 Hair follicle structure

Several studies described the physical and chemical properties of hair, its anatomy and physiology (Powell et al., 2002; Robbins, 2002). Although the hair shaft structure still remains unclear (Hakey, 1993; Huestis and Cone, 1998). Hair shaft is not an homogeneous fiber, but consists of keratinized cells glue by the cell membrane complex that together compose three concentric structures: 1) the cuticle, the outer covering of the hair; 2) the cortex, the largest portion of the hair and imparts strength to the hair shaft; and 3) the medulla, the central area of the hair and not always present (**Figure 2**). The pigmented cortex is responsible for the stretching stability and color composition, whereas the 5-10 layers of shingle-like cells of the non-pigmented cuticle is responsible for high chemical and physical resistance and shine (Pragst and Balikova, 2006). Hair originates from the hair follicle located 3-5 mm below the skin surface. The base of the hair follicle consists of the dermal papilla, which is richly supplied by blood vessels and sensory nerves. Within the hair papilla resides the hair bulb that is surrounded by the germination center formed by matrix cells (keratinocytes and melanocytes) that give rise to the different hair shaft layers. The rapid matrix cell mitosis forces a migration of the upper zones into the direction of the hair root mouth. Cell development occurs differently for

the cortex and cuticle. Cortex cells change from spherical shape at the germinative level to a spindle-like form. Protein filaments are synthetized, they fill the cell and fuse them together. Cuticle cells originates from matrix cells of the outer sphere of the papilla. These cells change to a shingle-like structure and contain amorphous protein. In brief, the hair cell membrane consists of protein and protein-lipid complex originating from previous cells. For this reason is supposed that CORT, due to its lipophilic nature, could be incorporated into the hair shaft.

Hair growing cycle is composed of the anagen (active growing), catagen (transition) and telogen (resting) stages but is still is difficult to assess the hair growing rate. This is due to relevant differences both in the proportions anagen/telogen hair and in growth rate from various anatomical sites. Both parameters are dependent on: race, sex, age and state of health. Generally in literature, is widely accepted that in humans scalp hair growth ranges 0.6-1.4 cm per month (Pecoraro and Astore, 1990; Pragst et al., 1998) and the preferred area on the human scalp to collect hair sample is vertex posterior where the proportion of telogen hair is lowest and growth rate is considered uniform (Pragst and Balikova, 2006).

2.5.2 Mechanisms of CORT incorporation and elimination

2.5.2.1 CORT incorporation into hair

The mechanisms involved in the incorporation of CORT into hair shaft is not yet known and well demonstrated. Consequently it is assumed that because free CORT and other steroid hormones are lipophilic substances, they enter in the hair shaft by passive/active diffusion from blood capillaries into growing cells over a length of 1.2 mm to 1.5 mm between the level of matrix cells and end of the keratinization zone of the hair follicle, so during its formation (**Figure 2**). This period would correspond to a timetable of CORT exposure of about three days (Pragst and Balikova, 2006). In this scenario hair CORT is hypothesized to reflect the integrated free fraction rather than the total CORT concentration in plasma (Davenport et al., 2006; Russell et al., 2012). Additionally, another pathway of incorporation of free CORT into hair is from the surrounding tissue (Meyer and Novak, 2012).

Once the growing shaft emerges from the scalp, it is coated with sebum originating from the sebaceous gland along with the sweat secreted by nearby eccrine glands (Raul et al., 2004; Pragst and Balikova, 2006). Early studies in humans demonstrated that CORT could diffuse from the bloodstream into both sebum and sweat (Cook and Spector, 1964; Jenkins et al., 1969). Thus, it is supposed that there could be some external contamination of CORT from these fluids onto the outside of the hair shaft after its formation (**Figure 2**).

Externally, it was demonstrated that drugs can deposit onto the hair shaft from the environment through smoke, pollution, physical contact and chemicals (Henderson, 1993; Cone, 1996; Gow et al., 2010). Regarding our animals, this external contamination can potentially due to secretions containing CORT as: blood, saliva, urine and feces.

Recently, the skin has been considered as an "*endocrine organ*" and the hair follicle a potential source of CORT (Slominski, 2005). In 2005, Ito et al. demonstrated the existence of a functional HPA-like system in human hair follicles in an in vitro organ culture. The follicle was able to synthesize CHR, ACTH, CORT, and receptors for these signaling molecules. CORT was founded in culture medium and leading a negative feedback on CRH synthesis. Moreover, recent studies reported that human hair on the arm displayed a rapid increase in immunoreactive CORT after challenge with a cold pressor test (Sharpley et al., 2009; 2010). These results suggest the possibility of a local stress response in the skin that could increase hair CORT concentrations but additional studies are needed.

In conclusion, It has been just assumed that hair glucocorticoids reflect free rather than total plasma CORT (Meyer and Novak, 2012; Russell et al., 2012) and evidences are still scarce. Furthermore, a demonstration that hair CORT accurately reflects central long term HPA activity that is not influenced in a transitory way by the peripheral one, is still uncertain.

2.5.2.2 CORT elimination

One of the most important methodological debate pertains to whether CORT concentration remain constant in the matrix and along the length of the hair shaft. Forensic scientists described that hair washing, shampooing, use of cosmetic treatments and the exposure to water and to UV radiation could negatively impact hair CORT levels in humans (Hamel et al., 2011; Li et al., 2012). Otherwise, in wildlife animals or captive animals living in outdoor enclosure UV radiation and water are considered potential sources of CORT elimination from hair (Meyer and Novak, 2012).

However, this problem arose from results of segmental hair analyses of CORT levels in humans examined by Kirschbaum et al. in 2009. This study examined hair CORT concentration along the hair shaft of nine nulliparous women. Hair samples of 18 cm length were collected from each woman and divided into six 3 cm sections. In the first segments, CORT concentration decreased ($P<0.0001$), 30-40% each time, reaching asymptotic-like level in the final two most distal segments. The authors suggested that this decrease was due to environmental damage of distal hair segments and advised using only the most proximal 1-6 cm of hair to assess systemic CORT concentration. Gao et al., (2010) confirmed similar results with segmental hair analysis collected from five people. Instead, Thomson et al. (2010) and Manenschijn et al. (2011) did not found in their investigations significant differences in CORT concentration along the length of the hair shaft. Moreover, CORT did not vary in

distribution in animals studies (Davenport et al., 2006; Bennett and Hayssen, 2010; Macbeth et al., 2010) even if these results could not be translated to the human condition, as the hair length and the hygiene in animals are considerably different from humans (Russell et al., 2012).

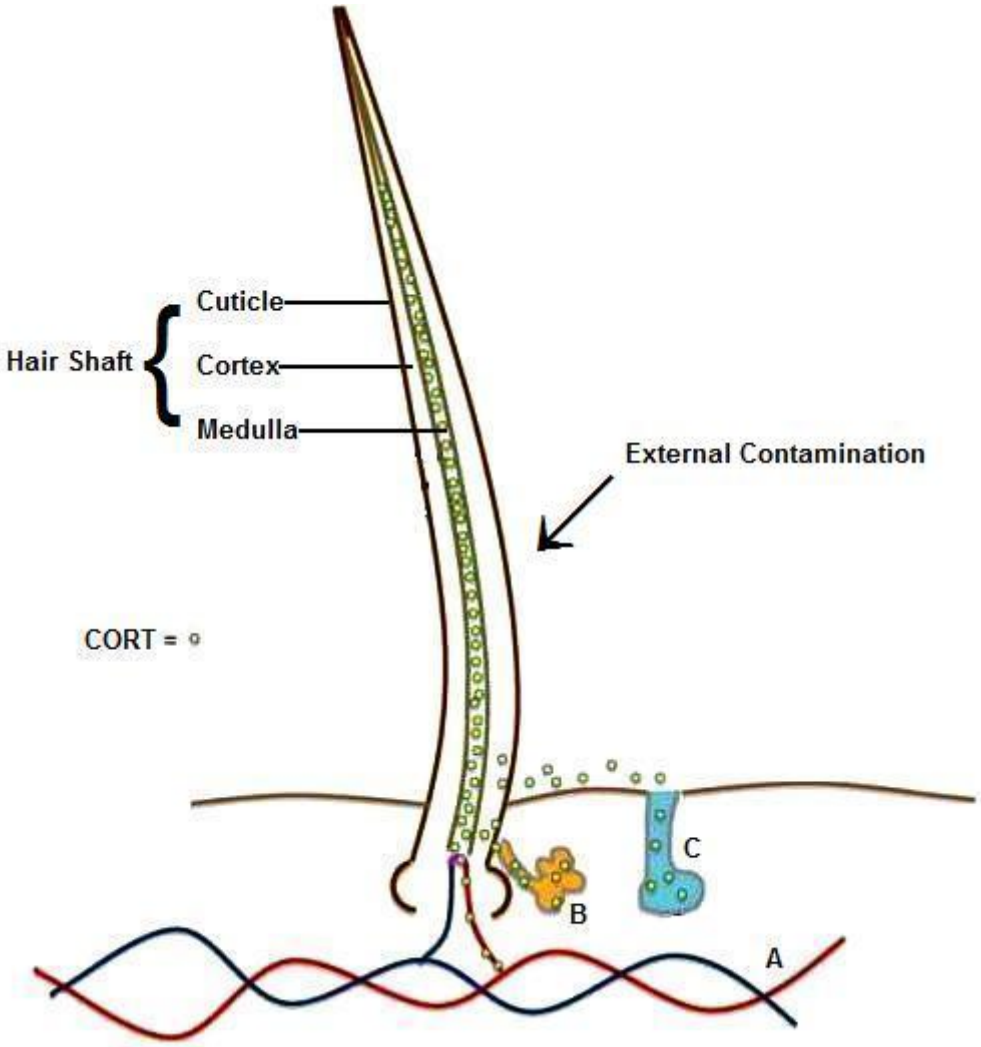

Figure 2. CORT incorporation into hair via **A**) Blood **B**) Sebum **C**) Sweat.

2.6 Methodological considerations

Before starting an experimental study using hair CORT as biomarker of HPA axis activity, investigators need to consider several issues regarding the specie and to obtain reliable results. Each of them will be discussed in this section.

2.6.1 Hair sampling and amount problem

The CORT is supposed to be incorporated from blood into the hair shaft during the anagen (active growing hair) and we can force this phase performing shave. As previously explained, the timing and pattern of hair growth is not uniform and the inter-body region variability is greater than the intra-body one. Thus, it is important to keep the body area of sampling constant across subjects and during repeated sampling procedures and using a "*shave-reshave method*" to establish a known timeline of CORT incorporation. Prior studies in humans analyzed hair samples from the vertex posterior of the head because this area has the lowest coefficient of variation (15.6%), as compared to hair sampled from other sites (30.5%) (Sauvé et al., 2007). For rhesus monkeys and captive caribou, hair was collected from the nape of the neck because this body region is relatively uncontaminated from self-grooming (Davenport et al., 2006; Ashley et al., 2011; Meyer and Novak, 2012). In domestic and farm animals hair was sampled from the ischiatic region or the costal side (Bennett and Hayssen, 2010; González-de-la-Vara et al., 2011), while in small rodents it was shaved all

the animal's back in order to obtain a large amount of hair (Martin and Réale, 2007; Karin et al., 2012). Hair should be always shaved close to the skin rather than plucked as to avoid including follicles in the sample that are capable of producing CORT in response to CRH stimulation (Gow et al., 2010; Meyer and Novak, 2012) and to prevent possible blood contamination

Amounts of hair obtained vary widely across studies, from 5 or 10 mg in humans (Stalder et al., 2010; Karlén et al., 2011) to 250 mg in rhesus monkey studies (Davenport et al., 2006). For forensic purposes, it is recommended to collect at least two separate hair samples. One sample must be enough for the analysis including confirmation and repetitions. The second hair sample should be left untouched if needed for future use (Pragst and Balkova, 2006). Obviously, this could be a problem in wildlife conditions or in laboratory animals medicine where we could not be always able to collect large amount of hair.

2.6.2 Storage requirements

An advantage of hair matrix is the stability of CORT over longer periods of time and that could be detected after years of storage. In fact, CORT can be even extracted from hair over thousands of years old (Webb et al., 2011). However, samples should be stored under dry and dark conditions at room temperature or at 4 °C. A suitable described procedure is to wrap the dried hair sample in aluminum foil prior to placement in a paper envelope (Pragst and Balikova, 2006). Plastic bags are accepted but firstly being careful to cover the

sample with aluminum because of a possible contamination by softeners that could extract lipophilic substances from hair.

2.6.3 Color, Age, Sex and Genetic as possible confounding factors

In forensic studies, hair color is considered an important factor that influences the incorporation and retention of drugs and other xenobiotics in hair (Kidwell and Smith, 2007). Dark hair is supposed to retain more drugs than light hair among different individuals (Rothe et al., 1997; Kidwell and Smith, 2007). Melanin is considered to be an important binding site for xenobiotics and different quantities and types of melanin in colored hair are responsible for observed patterns (Rollins et al., 2003). Predominant color patterns may vary with age, geographic location, sex and season, making hair color a potentially important confounding factor (Pearson, 1975; Schwartz et al., 2003). With respect to natural hair color in humans, neither Raul et al. (2004), Sauvé et al. (2007) nor Manenschijn et al. (2011) detected significant differences in CORT concentration among blond, brown or black hair. In animals, two bears studies (Macbeth et al., 2010; Bechshoft et al., 2011) revealed that hair CORT concentration were not affected by color. However, there are few research suggesting that melanin content could impact how CORT is sequestered into hair. In Bennet and Hayssen (2010), hair pigmentation showed a significant influence on CORT concentration, with black (eumelanin), agouti (mixed

melanin content), and yellow (phenomelanin) hair showing an ascending hair CORT concentration (r= 0.47; P= 0.001). Recently, Yamanashi et al. (2013) revealed that CORT in captive chimpanzees varied based on source body part and hair whiteness. Actually, this relationship is still poorly understood and future studies should consider this possible source of variation.

In regards to gender variability, few studies in animals reported no significant relationship between hair CORT and sex and age (i.e.: Bennet and Hayssen, 2010; Macbeth et al., 2010). However, post-natal development or pregnancy could be limiting factors. In humans, Kirschbaum et al. (2009) demonstrated that hair CORT concentration was significantly elevated in the third trimester relative to the first trimester and to non-pregnant control ($t(1,120)=4.77$; $P<0.001$). D'Anna-Hernandez et al. (2011) also founded CORT concentration elevated in the third trimester relative to the first trimester ($t=4.1$; $P<0.001$) and decreased again in the postpartum period ($t=2.9$; $P= 0.004$). It's also important to consider that the HPA axis activity (hyper- or hypo-secretion) may vary in relation to age, anxiety and social situation and could affect final results. For example, one human study showed how females had a heightened stress response to pharmacologically induced activation of the HPA axis than men. Conversely, men had an increased HPA response to mental stress (Uhart et al., 2006) but it is not yet demonstrated if the variability of stress response between men and women could affect hair CORT levels.

In animals studies, early post-natal development is a period of both normative changes in HPA activity and sensitivity of the system to different rearing environments. Like in plasma CORT, hair CORT levels are higher during early post natal development and gradually decline over the first several years of life in a number of species (Dettmer et al., 2012; Laudenslager et al., 2012; Feng et al., 2011; Fourie and Bernstein, 2011; González-de-la-Vara et al., 2011). Lastly, it has been reported significant genetic contributions to variation in hair CORT level in the baseline environment and that hair cortisol levels accurately reflect known phylogenetic related pattern (Fairbanks et al., 2011; Fourie and Bernstein, 2011).

2.6.4 Washing Procedure

In accordance with Bechshoft et al. (2011), there are two fractions of hair CORT: 1) fraction containing external CORT deposited by sweat, sebum and external contaminants (i.e.: dust, drugs and pollution) contributing to poor test results and analytical noise; 2) fraction containing CORT incorporated from blood into the hair shaft and that can only be extracted by stronger treatment of the sample. Hence, it is suitable to discriminate in the procedure two phases: the external washing procedure and the CORT extraction procedure.

The solvents used for external hair shaft decontamination should remove just external impurities and not extracting CORT from the hair matrix. However, in literature there is no general accordance with respect to the hair washing

procedure suggesting that requirements for an effective washing protocol might be specie-specific. This may relate to hair damage, amount and type of contamination or hair composition and resilience among different species (Harkey, 1993; Cone, 1996; Kidwell and Smith, 2007). Several studies report that multiple short washes with methanol or isopropanol are effective in removing visible contamination and measurable CORT from the animal hair and may provide a starting point for work in other species (Davenport et al., 2006; Bennett and Hayssen, 2010; Macbeth et al., 2010; Ashley et al. 2011; Bechshoft et al., 2011; Fairbanks et al., 2011). Even if Davenport et al. (2006) demonstrated that two brief washes with isopropanol remove only 5-10% of total CORT content in monkey hair and even after three consecutive washes the CORT concentration in the wash extract reached a plateau, some investigators still advise to not wash hair samples before processing (Thomson, 2008; Gow et al., 2010). Additionally, experimental studies in the forensic drug field indicate that external contaminants may penetrate the hair shaft deeply when hair is wet (Wang and Cone, 1995; Kidwell and Blank, 1996; Kidwell and Smith, 2007). Water expands the hair cuticle, facilitating permeability and providing a medium for diffusion and once the hair shaft is breached, contaminants may become tightly bound in the hair matrix and impossible to remove with a specific protocol (Kidwell and Blank, 1996). In contrast, substances placed on hair when dry are relatively easily removed (Kidwell and Smith, 2007).

The validity of these approaches is yet not clear. However, before starting a study, it is advisable to apply different trials procedures for a specific specie.

2.6.5 CORT Extraction Procedure

In several studies, hair samples are typically processed after washing and drying by mincing into small fragments (~1-5 mm long) or by grinding into a powder using a ball mill or a mini-bead beater to improve the solvent extraction activity. Pragst and Balikova (2006) discourage this latter approach in humans because generally results in loss of sample material and does not improve the extraction process. Contrarily, preliminary studies with monkey hair showed significantly greater CORT recovery after grinding compared with mincing, approximately 3.5 times more. Further, there was a greater variability in the results obtained from minced hair, with an inter-assay CV of approximately 24% compared to the 6.5% CV for powdered hair (Davenport et al., 2006). As an organic solvent, methanol is commonly used because has the dual advantage of causing swelling of the hair, facilitating analyte liberation by diffusion, and solubilizing steroid hormones such as CORT (Meyer and Novak, 2012). This organic solvent is usually added for in each sample and after its evaporation, the extract is reconstituted in an appropriate medium and analyzed for its CORT content.

2.7 CORT assay methods

Hair CORT can be quantified by several assay methods and to date the most used are: enzyme-linked immunosorbent assays (ELISA) or enzyme immunoassays (EIA), high performance-liquid chromatography-mass spectrometry (HPLC-MS) and radio-immunoassays (RIA).

2.7.1 Mass spectrometry (GC/MS and LC/MS)

These methods are highly specific and sensitive techniques. Gas chromatography/mass spectrometry (GC/MS) is widely used in human hair analysis to detect drugs or metabolites with a limit of detection around 0.03 ng/mg (Pragst and Balikova, 2006). High performance-liquid chromatography-mass spectrometry (HPLC/MS or LC/MS) is the gold standard to quantify CORT in hair (Crimele et al., 2000; Gaillard et al., 2000; Raul et al., 2004; Kirschbaum et al., 2009). Generally, hair samples are washed as described above even if methylene chloride replaces isopropanol. The amount of powdered or minced hair to analyze should be between 30 and 100 mg and is incubated in 2 ml of Soerensen buffer. The extraction recovery reported for this method was 74% and intra-day coefficient of variation (CV) value of 11% (Raul et al., 2004). The lower limit of detection (LOD) and quantification was found to be 1 and 5 pg/mg, respectively. While HPLC/MS is an extremely accurate method of CORT analysis, this assay is expensive, needs expert technicians and remains limited to a small number of forensic laboratories.

2.7.2 ELISA (or EIA)

This method is widely used because offers a good sensitivity, quick results and low cost. The hair sample amount suggested should be between 8-50 mg (Davenport et al., 2006; Davenport et al., 2008; Martin and Reale, 2008; Kirschbaum et al, 2009) and incubated in 1.5 ml of methanol for 16 h at 50 °C. for CORT extraction. The sample is analyzed in duplicate using a commercially available salivary enzyme immunoassay kit from *Alpco Diagnostics* (Koren et al., 2002; Klein et al, 2004; Sauvé et al., 2007; Thomson, 2008; Van Uum and Sauve, 2008; ; Gow et al., 2009; Thomson et al., 2009) or other manufacturers, such as *Salimetrics* and *Assay Designs* (Davenport et al., 2006; Davenport et al., 2008; Martin and Reale, 2008). The intra- and inter -day CV from *Alpco Diagnostics* was 3.8% and 8% (Gow et al., 2010) and other studies demonstrated these values to be at least below 11% (Sauvé et al., 2007; Davenport et al., 2006; Van Uum and Sauve, 2008; Kramer et al., 2009) demonstrating the repeatability of this technique. According to *Alpco Diagnostics*, the EIA kit has minor cross reactivity with compounds such as corticosterone (7.6%), prednisolone (9.53%), and GCs below 1% (Davenport et al., 2006). The LOD as reported by *Alpco Diagnostics* is 1 ng/ml but for salivary CORT. Thomson (2008) reported 20 pg/mg to be the lowest LOD for hair CORT while in Davenport et al. (2006) was 0.9 pg/mg. The extraction recoveries were 87.9%, 88.9% and 87.4% for hair samples spiked with decreasing concentration of CORT (12) while serial dilution experiments by Thomson (2008) revealed an

average recovery of 109.5% for a standard hair sample and 93.5% for a standard chemical sample (Thomson, 2008).

2.7.3 Radio-immunoassay (RIA)

Hair samples are prepared in a similar way as previously described. Focusing on the RIA methods used in two studies, 25-50 mg minced hair sample should be incubated with 5 ml of methanol (Accorsi et al., 2008) or with 3 ml of ether (Yang et al., 1998) to extract the steroids. The samples are incubated at 50 °C for 18 h and the residue remaining after dryness is reconstituted in 0.2-0.6 ml of PBS. 100 µl of ^{3}H-cortisol and µl of an anti-cortisol antibody are added to 100 µl if the CORT extract solution (Accorsi et al., 2008) and after 18 h incubation at 4 °C, free CORT is separated from bound CORT by adding 1 ml of charcoal solution 1%, 15 min incubation at 4 °C and a centrifugation at 4000 X g for 4 min at 4 °C (Accorsi et al., 2008). The RIA method reported a sensitivity of 0.26 pg/mg, a specificity of 100% for CORT and intra- and inter-assay CV was 6.8% and 9.3%, respectively (Accorsi et al., 2008). Yang et al. (1998) revealed an inter-assay CV below 10% and cross reactivity less than 2.4% for other hormones. The RIA method is highly specific and sensitive but expensive, it requires special precautions and expert technicians.

While the methods described above could be negatively influenced by external contaminants, they all confirmed CVs around or below 10% and a

minimum variation of hair amount required, being 10 mg for the ELISA, 30 mg for the LC/MS and 25 mg for the RIA. Besides, ELISA method was validated using LCMS/MS by Kirschbaum et al. (2009) and nowadays is widely used in laboratories. However, even if it has good sensitivity, there is some variation in results seen among similar ELISA methods and its accuracy is still debated. In conclusion, it is better to consider as "gold standard" methods of hair analysis the LC/MS and RIA because they provide both higher sensitivity and specificity.

2.8 Relationship between hair CORT concentration and CORT in other matrices

Recent studies examined potential relationships between hair CORT and CORT concentration in the other matrices such as saliva, feces and urine. In comparing hair and salivary CORT in the same individuals, the data ranged from no significant correlation in two human studies (Sauvé et al., 2007; Steudte et al., 2011) to a 0.8 correlation in adult male rhesus macaques but in a stable environment (Davenport et al., 2008). Recently, two human studies reported statistical significant correlations of 0.3-0.4 between hair and salivary CORT(van Holland et al., 2011; Xie et al., 2011). These finding suggest that long-term HPA activity as assessed by hair CORT concentration is related to short-term activity assessed by salivary CORT under some conditions. However, hair CORT was demonstrated to be more closely related to urinary and fecal CORT excretion. A significant correlation ranging from 0.33 for 24-h urinary CORT excretion in humans (Sauvé et al., 2007) to 0.67 and 0.90 for fecal CORT excretion in domestic animals were reported (Accorsi et al., 2008). These results suggest that CORT hair approach should be thought as complementary to measurements of salivary and/or plasma CORT but not yet as replacement for such measurements.

Property	Blood	Saliva	Urine	Feces	Hair
Results affected by stress of sampling procedure	YES	YES	NO	NO	**NO**
Storage conditions	Spinning, Refrigeration, Freezing	Refrigeration, Freezing	Refrigeration, Freezing	Refrigeration, Freezing	**Room Temperature**
Time periods of Cort represented	Single point	Single point	12-24 h	24-48 h	**Weeks, Months, Years**
Total or free Cort	Total Cort measured	Free Cort measured	Free Cort measured	Free Cort measured	**Free Cort measured**
Reference ranges in literature?	YES	YES	YES	YES	**NO**

Table 1. Comprehensive properties of different matrices where CORT can be analyzed.

2.9 Application of Hair CORT in research

Hair CORT research is rapidly expanding, with research in humans, non-human primates, domestic and farm animals, wildlife animals and many other species, which results in a wide range of topics, methods and research questions that are difficult to unify (**Table 2**). For example, information about the sample and analyzing methods are prerequisite for possible comparison between different articles. Otherwise, this comparison is difficult as there are several methodological differences between research groups. The washing procedure, the mincing of hair and the analysis after CORT extraction differs between many laboratories and these differences are partly caused by the still unresolved issue how exactly CORT is incorporated into hair.

Mainly this technique has been used in human research to investigate the influence of major life stressors on long-term HPA activity. They include individuals patients hospitalized with acute myocardial infarction compared with control patient (Pereg et al., 2011), daily workers compared to shift workers (Manenschijn et al., 2011), individuals who were unemployed for 12 months compared with those who had jobs (Dettenborn et al., 2010), alcohol-dependent individuals undergoing withdrawal compared with control subjects (Stalder et al., 2010), and healthy newborns compared with infants in intensive care unit (Yamada et al., 2007). These studies demonstrated that hair CORT concentration were elevated in subjects undergoing significant stress compared

with control groups or with the same subjects before stress imposition. Among the human clinical applications of hair CORT are: the determination and monitoring disease progression of long-term changes in CORT levels in patients with Cushing's syndrome or adrenal insufficiency (Thomson et al., 2010; Manenschijn et al., 2011), and neuropsychiatric disorder characterized by dysregulation of the HPA axis (Steudte et al., 2011; Steudte et al, 2011b; Dettenborn et al., 2012).

In laboratory animals this new biomarker of HPA axis activity was applied just in few chronic stressors studies regarding rhesus macaques, bonnet macaques and vervet monkeys subjected to relocation stress (Davenport et al., 2006; Davenport et al., 2008; Fairbanks et al., 2011; Laudenslager, et al., 2012). Hair CORT was used in wildlife and environmental conservation studies (Macbeth et al., 2010; Bechshoft et al., 2011) and are expected some investigations concerning the assessment of stress in wild-living populations. Moreover, further research is needed to ascertain the genetic and environmental factors that contribute jointly to temperament and HPA activity. Recently, hair CORT levels were found to be: lower in vervet monkeys with a high novelty-seeking animals (Laudenslager et al., 2011); hair CORT in adult dogs was positively related to behavioral reactivity after exposure to the sounds of thunderstorm (Siniscalchi et al., 2012); positively correlated with a docility index in eastern chipmunks (Martin and Réale, 2008); and with agonistic behaviors in free-roaming female cats in Tel Aviv (Finkler and Terkel, 2010).

Authors	Topic	Animals	Hair Sample	Method	Results
Accorsi et al., 2008	Cortisol determination in hair and feces	Domestic cats and dogs	n= 27 cats: 19 female; 8 male (2- 10 y) n= 29 dogs; 8 female; 21 male (1- 7 y)	1- 3 mm minced hair Methanol extraction RIA	Significant positive association observed in both species between the concentrations of cortisol determined in hair and feces
Ashley et al., 2011	Cortisol concentration in feces and hair following ACTH challenge	Adult captive caribou	n= 6 male n= 6 female	3 min methanol (x3) Powder using ball mill Methanol extraction EIA Kit	Hair cortisol concentration unaffected by a single ACTH challenge
Bechshoft et al., 2011	Cortisol concentration in hair	East Greenland polar bears	n= 10 males (6-19 y) n= 7 female (3- 19 y)	3 min isopropanol (x2) Powder using ball mill Methanol extraction EIA Kit	No significant correlation between cortisol concentration and age (p= 0.81) or sampling year (p= 0.11) Females higher mean cortisol concentration than males (females mean: 11.0 pg/mg, males: 7.3 pg/mg; p= 0.01)
Bennet and Hayssen, 2010	Cortisol concentration in hair and saliva	Domestic dogs	n= 48; 28 female; 20 male (9- 11 y)	3 min isopropanol (x2) Powder using ball mill Methanol extraction EIA Kit	Salivary and hair cortisol concentrations positively correlated (P= 0.001) Black dogs less cortisol than nonblack dogs (P= 0.039) in hair, but not saliva Average amount of cortisol not differ between proximal and distal hair sections (P= 0.348) Difference in cortisol concentrations among hairs of different colors from individual dogs (P= 0.001)
Davenport et al., 2006	Cortisol concentration in hair and saliva after relocation	Captive rhesus macaques	n= 20 male (8- 22 y)	**3 min Isopropanol (x2)** **Powder using ball mill** **Methanol extraction** **EIA Kit**	Proximal and distal segments of hair do not differ in cortisol concentration Prolonged stressful experience produces an increase in hair cortisol Hair cortisol concentration correlated with salivary cortisol levels (r= 0.797; p< 0.001)

Dettmer et al., 2012	Physiological and behavioral adaptation to relocation stress	Differentially reared rhesus monkeys	n= 61; 33 male; 28 female (from birth to 2 y)	3 min Isopropanol (x2) Powder using ball mill Methanol extraction EIA Kit	Elevated hair cortisol early in life as biomarker for the later development of anxious behavior in response to a major life stressor
Fairbanks et al., 2011	Heritability and genetic correlation of hair cortisol in low and higher stress environments	Vervet monkeys	n= 226 adult female (3- 18 y)	3 min Isopropanol (x2) Powder using ball mill Methanol extraction EIA Kit	Levels of cortisol in hair increased significantly from the baseline to the post-move environment. Hair cortisol levels heritable in both environments (h(2)=0.31) High genetic correlation across environments (rhoG=0.79)
Feng et al., 2011	Changes in cortisol and behavior after maternal separation	Rhesus monkeys	Mother-reared (n= 22) Peer-reared (n= 13); 1.5- 2 y	3 min Isopropanol (x2) Powder using ball mill Methanol extraction EIA Kit	Rearing condition affected cortisol levels. Hair cortisol levels decreased in the PR compared with the MR at 2 y of age
Finkler and Terkel, 2010	Cortisol levels and aggression	Cats in urban social groups	n= 66 adult (> 1 y) female (29 intact and 37 neutered) Hair collected from 15 (of the 29) intact and 36 (of the 37) neutered female	2 mm minced hair Methanol extraction RIA	Neutered females reduced aggressiveness and reduced cortisol levels compared to the intact females Intact females with more aggression had higher cortisol levels compared to the less aggressive intact females
Fourie and Bernstein, 2011	Hair cortisol concentration phylogenetic and age related differences	Wild caught vervet monkeys (*C. aethiops*) and captive Guinea baboons (*P. hamadryas papio*)	n= 19 Guinea Baboons (1- 6 y) 8 female; 11 male n= 49 Vervet monkeys (0.5 – 2.5 y) 29 female; 20 male	3 min Isopropanol (x2) 1- 2 mm minced hair Methanol extraction EIA Kit	Infants exhibit hypercortisolism Hair cortisol declines from infancy through juvenile phases Hair cortisol levels accurately reflect known phylogenetic pattern
González-de-la-Vara et al., 2011	Effects of ACTH challenge and age on hair cortisol concentration	Dairy cattle (Cow and calves)	Holstein Cows (n = 6; 2 y) Holstein Calves (n = 6; 15 d)	Methanol washing protocol 2 mm minced hair Methanol extraction RIA	Concentrations greater in calves than in cows and greater in white hair than in black hair Cortisol accumulated in bovine hair after ACTH challenges but the concentration was affected by age and hair color
Keckeis et al., 2012	Radio-metabolism study of hair cortisol concentration	Guinea Pigs	n= 8; 4 female, 4 male (4.2- 4.5 months)	10 min n-hexane (x2) 5 ml methanol (x2) No minced and no powder hair Methanol extraction HPLC + EIA	Small amounts of administered radioactive GCs are deposited in hair Measurement of large amounts of unlabeled GCM suggests local production of glucocorticoids in hair follicles

Koren et al., 2002	Cortisol concentration in wildlife animals	Rock Hyrax	n= 10; adult male	3-4 mm minced hair Methanol extraction EIA Kit	Determination of cortisol and testosterone concentration
Laudenslager et al., 2011	Hair cortisol reflecting a novelty seeking phenotype	Vervet Monkeys	n= 230; female (3- 18 y)	3 min Isopropanol (x2) Powder using ball mill Methanol extraction EIA Kit	High hair cortisol levels are consistently more cautious and inhibited in response to novelty compared to females with low hair cortisol levels
Laudenslager et al., 2012	Hair cortisol concentration related to behavior	Bonnet macaques (*Macaca radiata*)	n= 22; 9 female, 13 male (25.0 ± 5.7 m)	3 min Isopropanol (x2) Powder using ball mill Methanol extraction HPLC	Low average cortisol levels promote novelty seeking, while high average levels inhibit novelty seeking behavior
Macbeth et al., 2010	Hair cortisol concentration in wildlife animals	Grizzly bears (*Ursus arctos*)	n= 171	3 min Methanol (x5) Powder using a mixer mill Methanol extraction EIA kit	Hair cortisol varies with hair type, body region, and capture method. It is not influenced by color, age, sex, environmental exposure, or prolonged laboratory storage and does not vary along the hair shaft
Martin and Réale, 2008	Assessing the relationship between cortisol accumulated in the hair and both temperament and frequentation by humans	Eaastern chipmunks (*Tamias striatus*)	n= 23	1 h Ether 3- 4 mm minced hair Ether extraction EIA kit	Hair cortisol increased in docile animals, but was not related to human frequentation
Siniscalchi et al., 2012	Cortisol levels in hair related to behavioral reactivity	Domestic dogs of various breed	n= 14; 8 female 6 male (2- 13 y)	1- 3 mm minced hair Methanol extraction RIA	Levels of cortisol in hair appear to reflect the dog's chronic state of emotional reactivity, or temperament
Yamanashi et al., 2013	Hair cortisol analysis	Captive chimpanzees (*Pan troglodytes*)	n= 26; 10 female, 16= male (11- 41 m)	2 min Methanol (x3) Powder using Precelly 24 Methanol extraction EIA kit	The results reveals that cortisol variation is based on source body part and hair whiteness

3. AIMS OF THE STUDIES

The main objective of this work was to introduce and to validate a new method to analyze hair CORT concentration in both livestock and laboratory animal fields, to improve some aspects of extraction and assays procedures and to find an answer to some questions and gaps about this novel measure that need to be addressed. The goal is to make hair CORT: 1) a more practical and accurate biomarker in chronic stress and animal welfare investigations; and 2) a more attractive and accessible technique for researchers involved in veterinary and biomedical research. In order to do that, studies using commercial sows and Sprague-Dawley rats were performed, and the specific aims of these two studies were:

1. To develop and validate a reliable non-invasive method for measuring hair cortisol concentration in pigs and corticosterone in laboratory Sprague-Dawley rats.
2. To give useful information on hair cortisol concentration in commercial sows and to analyze long-term changes of this hormone in standard breeding condition.
3. To provide evidence that supports the hypothesis that hair glucocorticoid is representative of the free rather than the total fraction of circulating glucocorticoids.

4. To demonstrate that hair corticosterone in Sprague-Dawley rats accurately reflects long term HPA axis activity.
5. To show hair corticosterone concentration changes in response to chronic stress manipulations and chronic ACTH administration.

4. HAIR CORTISOL CONCENTRATION IN SOWS DURING TWO REPRODUCTIVE CYCLES IN STANDARD BREEDING CONDITION

4.1 Materials and Methods

4.1.1 Animals

In this study, thirty randomly selected, naturally synchronized hybrid Goland sows with a mean parity of 4.68 ± 3.1 (mean ± SD) from a breeding center of four hundred sows located in the Emilia Romagna region, Italy were used. This research was carried out during different seasons monitoring two successive and naturally synchronized reproductive cycles. Breeding management was a batch farrowing system every third week leading to a natural synchronization by weaning. The sows were bred accordingly with the current Italian legislation implementing Council Directive 2008/120 EC concerning the protection of pigs.

4.1.2 Experimental time points

During intensive farming, the standard sow reproductive cycle is repetitive under physiological conditions. Therefore we can consider that the duration of the different periods is almost the same. Generally, pregnancy lasts 114 days (± 2 days) and lactation (as well as lactation anestrous) ends since

piglets are weaned at 25-28 days of age. The sow returns to heat within the first week after weaning and goes through the Artificial Insemination (AI) and the ultrasonographic diagnosis to confirm pregnancy can be achieved around 35-40 days after the AI. The sow reproductive cycle starts with the AI and ends up at weaning.

From the pregnancy diagnosis until 5 days before the expected date of birth (about 9 weeks), the sows were housed in collective pens with a free access to an outdoor gestation area. Then, the animals were reallocated to the farrowing room and individually housed in the farrowing boxes until weaning. The lactation period was about 4 weeks. After weaning, the sows were moved to the mating house where they were individually housed in single pens. The animals were expected to come into estrous 5 days after weaning. The sows stayed in the mating house until 40 days after the AI, when pregnancy was confirmed. Then, they were moved again to the outdoor gestation area and re-grouped into collective pens.

Hair samples were collected during three phases of the reproduction in two successive cycles, i.e. Before Delivery (5 days pre-partum, BD1 - BD2), at Weaning Time (25-28 days post-partum, WT1 - WT2) and at diagnosis of the next pregnancy (70-75 days post-partum, Pregnancy Diagnosis, PD1 - PD2) (**Table 3**).

4.1.3 Room and environmental temperature

The gestation facility presented a negative pressure ventilation system whereas the sow farrowing facility included also a heating system. Room temperatures were recorded both in the gestation as in the farrowing facility through two data loggers (Model 175 H2-Testo, Lenzkirch, Germany). Environmental temperatures data were obtained from a previous historical record from a nearby weather station.

4.1.4 Hair growth measurement

Before starting the experiment it was defined the bristles growing rate. The rump body area of three sows was shaved using an electric grooming clipper. After one month we measured the re-growing hair (anagen phase) using a decimal Vernier caliper.

4.1.5 Hair sampling

The hair was collected shaving the animal rump as close as possible to the skin in an area of max 49 cm^2, alternately on the left and on the right side, at successive experimental time points, being careful not to shave hair from the area previously treated. Accordingly with forensic procedures, the hair was enveloped into an aluminum foil and then into a small plastic pouch bag, individually and correctly marked, and stored in a dark and cold chamber at 4 °C until the analysis.

4.1.6 Sample washing and extraction procedure

In order to validate the cortisol extraction procedure it was decided to conduct some preliminary trials. At the beginning, two consecutives water washes (50 ml of deionized water for 45 sec with gentle rotation) were needed to remove the hydrophilic organic contaminants deposited on the external hair shaft. The deionized water samples were extracted with methanol and analyzed by RIA. Cortisol was just found in the first sample (data not shown). The same hair samples were treated with two consecutive isopropanol washing procedures to remove the lipophilic organic substances and sebum deposited on the external hair shaft. The RIA analysis showed no cortisol detection in any isopropanol samples.

In brief, the optimized protocol was settled as follow: one deionized water washing procedure and two isopropanol washes. After water washing the samples needed to be dried at room temperature (RT) for about 1 hour. Then, 250 mg of bristles were placed in polypropylene tubes, and, as suggested in Davenport et al (2006), 5 mL of isopropanol were added. The samples were slowly shacked on a rotator for 3 min at RT, then centrifuged at 1500 x g for 1 min: The isopropanol was recovered and bristles washed once again, then air dried. The samples were powdered in liquid nitrogen using a mortar. 60 mg of sample was placed in a glass vial with 2 ml of methanol and incubated for 21 hours at RT with continuous rotation. After a fast centrifuge at 1500 x g for 5 min, 1.8 ml of methanol was recovered, transferred into a tube glass and

evaporated to dryness under gentle air-stream hood at 37 °C. The samples dried were stored at -20 °C for further analysis.

To obtain a sample pool for the inter and intra-assay RIA evaluations, 60 mg of powdered hair from each sow were pooled together, extracted with methanol, dried as previously described and analyzed in triplicate during each RIA.

4.1.7 Cortisol radioimmunoassay and validation of hair cortisol assay method

Cortisol concentration was determined using a validated RIA assay (Tamanini et al., 1983). The samples with dried extract were dissolved in 300 µl of an RIA-phosphate buffer (Na_2HPO_4 74.26 mmol/l, EDTA Na 12.49 mmol/l, NaN_3 7.69 mmol/l) containing 0.1% bovine serum albumin, pH 7.5, and shaken for 10 min; 100 µl were then analyzed. After adding 0.1 ml of 1,2,6,7-3143 H cortisol (30 pg/tube), and 0.1 ml of rabbit antiserum raised against cortisol (produced in our laboratory) (Tamanini et al., 1983) at a working dilution of 1:20,000, the samples were incubated overnight at 4°C. After incubation, 1 ml of charcoal-dextran solution (charcoal 0.25%, dextran 0.02% in phosphate buffer) was added to the tubes; after 15 min at 4°C, the tubes were centrifuged for 15 min at 3000 x g, the supernatant was decanted and radioactivity was immediately measured using a β scintillation counter (Packard C1600, PerkinElmer, USA). Cross reactions of various steroids with antiserum raised

against cortisol were tested: cortisol 100%, cortisone 5.3%, 11α-deoxycortisol 5.0%, corticosterone 9.5%, 20α-dihydrocortisone 0.4%, prednisolone 4.60%, progesterone <0.001% and testosterone <0.001%. Recovery was evaluated by adding known quantities of standard cortisol to the pooled hair samples which underwent extraction. In order to determine the parallelism among cortisol standards (dilutions of hormone ranging from 7.81 to 1000 pg/100 µl) and endogenous cortisol in swine hair, a pooled sample containing a high concentration of cortisol was serially diluted (1:1 - 1:16) with RIA buffer. Using the optimized procedures the mean percentage of recovery was 96.6 ± 2.2%. Assay repeatability was assessed by calculation of intra- and inter-assay CV for one pool of bristle extract with mean cortisol concentration of 50.2 pg/mg. Intra- and inter- assay CV were 2.76% (n= 5 determinations in triplicate) and 8.50% (n= triplicate determinations in 5 assays), respectively. The sensitivity of the assay was 4.81 pg/tube and was defined as the dose of hormone at 90% binding (B/B0). A high degree of parallelism was confirmed by a regression test (r^2 =0.99; $P<0.01$), demonstrating the specificity of the procedure for determining hair cortisol concentration in pigs.

4.1.8 Statistical analysis

Statistical analysis was carried out using Statistica version 10.0 (StatSoft, Inc. 2011). Data are presented as mean ± SEM. A descriptive statistical analysis was used to evaluate the data obtained for the sampled sows. In order to study the difference between the sampling points, data were analyzed with 1-

way analysis of variance (ANOVA) and the post-hoc Duncan multiple range test. To evaluate the effect of reproductive phase (PD vs BD) and season a two-way analysis of variance was used. We used a regression analysis to determine parallelism between authentic cortisol standards and endogenous hormones run in the same assay.

4.2 Results

The sows were monitored from February to November, during two consecutive reproductive cycles under common breeding and rearing conditions, previously described.

During the first reproductive cycle (BD1, PD1, WT1) samples were collected from thirty sows and during the second cycle (BD2, PD2) samples collected from the remaining twenty five sows, since 16,6% of them were sacrificed.

Until the last day of the experimental design, all the animals were healthy and no disease occurrences were recorded. The monthly mean room temperature ranged between a maximum in July (29 °C in the farrowing house) and a minimum in November (17 °C in the gestation house). Environmental temperature data during the experimental period are reported in **Figure 3**. The mean rate of hair re-growth (anagen phase) was 0.7 ± 0.2 cm/month.

Cortisol hair concentrations at different experimental time points are shown in **Figure 4**. The mean hair concentration of cortisol at PD1 and PD2 were 20.10 ± 1.95 pg/mg and 16.29 ± 2.15 pg/mg respectively. PD1 and PD2 were not significantly different although a tendency towards a reduction during the hot season was confirmed ($P<0.058$). PD1 and PD2 were both significantly higher ($P<0.001$) when compared to BD1, WT1 and BD2 mean values. The mean cortisol concentration at BD2 was significantly higher ($P<0.001$) in comparison to the BD1 and WT1 sampling times (10.48 ± 0.96 pg/mg vs. 5.17 ± 0.51 and 6.01 ± 0.47 pg/mg respectively). The two-way analysis of variance resulted in significant differences for reproductive phase ($P<0.00001$) and season ($P<0.005$). Instead the interaction between the reproductive phase and the season was not significant.

In order to study the distribution of subjects with different cortisol contents, the sows were divided into three categories on the basis of mean cortisol concentrations: 1 to 10 pg/mg, 10 to 20 pg/mg and more than 20 pg/mg (**Figure 5**). At WT1, all sows (100%) fell into the lowest category (1 to 10 pg/mg hair shaft), at BD1 and BD2, the majority (90.9% and 55.55%,respectively) were in the lowest category, and 9.1% and 44.45% were in the middle category (10 to 20 pg/mg) while only at PD1 and PD2 did the majority of the sows fall into the middle/high cortisol category (96% and 72.23%, respectively).

4.3 Discussion

This study aimed to fill the lack existing in pigs by developing a cortisol extraction procedure from bristles and a validation of the RIA method.

To this end, we followed a group of sows during different phases of their reproductive cycle to evaluate how a possible influence of several environmental and physiological conditions could act on hair cortisol levels.

In contrast with previous studies and with respect to the pre-analytical handling of samples, we found advantageous and useful to add a deionized water washing since pigs hair shaft is usually highly contaminated with external organic hydrophilic materials (i.e.: saliva, urine, feces, blood and scurf) containing unknown amount of cortisol. Highly reproducible results were obtained in preliminary trials using different amounts of the same samples applying this modified protocol.

Besides, even if few data are present in literature regarding swine hair cycle (Mowafy and Cassens, 1976; Watson and Moore, 1990), in this research, it was observed a very slow re-growth (anagen phase) of the hair (~0.7 cm/month) which was inside the thickness of the skin for at least 3-4 mm (Mowafy and Cassens, 1976). Therefore, we can assume that in pigs, hair samples could date back to at least 15 days earlier.

To evaluate if hair analysis could give us information regarding cortisol concentration over long-term periods, we settled two specific experimental time points: 1) BD time point (5 days Before Delivery) giving information about the previous period when pregnant sows were housed in groups, had social relationships and they could move in and out from the pen for at least 74 days (positive conditions with some acute stressors); 2) PD time point (at Pregnancy Diagnosis) corresponding to ~80 days of systematic stressors during which the animals were restrained in farrowing crates for parturition and lactation and were subsequently moved to single crates for AI.

Since in pigs, the plasma cortisol values, in physiological conditions, increase before and during shipment and slowly returns to a basal level at weaning (Whitely et al., 1984), we decided to analyze the hair collected at the weaning of the first reproductive cycle (WT1) and to get information about this specific time period. Our results demonstrated that, at WT1, the mean cortisol concentration did not differ with the BD1 period. We believe that this outcome was probably due to the fact that 25-28 days after delivery, the bristle sample collected did not shown yet the effects of the plateau in blood cortisol which occurred around farrowing. For this reason we did not repeat hair sampling at weaning during second cycle.

The same twenty five sows were hair collected again during the following reproductive cycle. Our data showed that before delivery in both cycles the mean cortisol level was notably lower than at pregnancy diagnosis time point,

indicating a clear influence of the reproductive phase. As previously reported by Hay et al. (2000), during early and mid-gestation, the mean plasma cortisol concentration (12.6 and 15.5 ng/ml) was significantly lower than in late pregnancy (19.95 ng/ml). Moreover, during farrowing, higher levels in plasma were described for at least 2 days by Osterlundh et al. (1998) but diminishing slowly and progressively during lactation (Biensen et al., 1996).

Between the two reproductive cycles no significant differences were detected at pregnancy diagnosis (PD1 and PD2 time points) however, at PD2, the content of cortisol tended to be decreased. Accordingly to literature, at pregnancy diagnosis (~65 days after farrowing) the hair cortisol concentration provided a good index about the periods of high plasma cortisol levels experienced by the animal during: late pregnancy, delivery and lactation. Moreover, the breeding condition (farrowing and single crate) are known to be more stressful during this phase, due to several chronic and intermittent stressors influencing all aspects of their life (EFSA, 1997; Kemp and Soede, 2012).

Although, before delivery (BD1 and BD2 time points) sows had experienced two months of free breeding condition, several social relationships and presumably fewer chronic intermittent stressors. Our results pointed out a diminished mean of hair cortisol concentration at both BD1 and BD2 compared to PD1 and PD2. However, the cortisol concentration at BD1 was remarkably lower than that one recorded at BD2. The reliability of these results in BD1 were

confirmed by similar outcomes obtained after one month at WT1, even if they represented different sampling and RIA analyses.

Our results confirmed the important influence of the season that is probably linked to different thermal and lighting conditions. In fact, the BD2 samples were collected at the end of November while BD1 during the middle of June. This situation could explain the different trends observed for cortisol values founded between PD1 and PD2. At PD2, in September, the hair cortisol concentration tended to be lower than in April at PD1, reflecting the plasma cortisol concentration of the sows during the previous months preceding the sampling. In fact, it has already been demonstrated that, when an adverse stressor is prolonged, the circulating cortisol concentration not only diminish (i.e.: during heat stress or crowding: Kattesh et al., 1980; Sutherland et al., 2006), but that adrenocorticotropic hormone (ACTH) challenges also give a more limited response (heat stress: Peltoniemi et al., 1997). Therefore we could affirm that, in sows, during the warmer months (May, June, July and August) the mean plasma cortisol concentration is reduced in regardless of the reproductive phase, and the opposite situation for colder months (October, November, February and March), allowing us to hypothesize a seasonal fluctuation of cortisol. Seasonal variations of the plasma cortisol concentration, as well as a seasonal variation in adrenal responsiveness to ACTH, have been described in unrestrained red deer (Ingram et al., 1999). Again circannual cortisol variations have also been found in humans (Matchock et al., 2007; Persson et al., 2008;

Miller et al., 2007), in goats (Alila-Johansson et al., 2003), in sheep (Ssewannyana et al., 1990), in horses (Cordero, 2012) and in squirrels (Boswell et al., 1994).

In swine, seasonal infertility is well known (Love, 1978; Love et al., 1993; Peltoniemi et al., 2000; Bassett et al., 2001; Einarsson et al., 2008) and is supposed to be correlated to the complex interaction between environmental factors which are mediated by a variety of endocrine signals. Fluctuations in the monthly farrowing rate are observed in the same period at different places in the two hemispheres (Northern and Southern) and also in Northern Italy, when in a recent survey on 18 sows, the highest percentages of sows not pregnant after AI was described in July, August and September (19.62%, 17.88%, 17.60% respectively) (Mazzoni et al., 2012).

From these important data, we wonder if this reduction of cortisol secretion during the hot season could be one of the several factors influencing the swine fertility. Thus, the physiological status, the breeding condition and the seasonality could be variables influencing the hair cortisol concentration in pigs. The present research allowed us evaluating separately the seasonality effect, whereas physiological status and breeding condition could not be evaluated independently. However, we suppose that hair cortisol could be also a sensitive index detecting circannual physiological variations of circulating cortisol.

With respect to individual animals, we observed a high proportion (more than 30%) of subjects with a mean of hair cortisol content higher than 20 pg/mg, with one animal peaking to 52 pg/mg only at the PD1 and PD2 time points. These specific results could not be ascribed to known conditions (parity, number of piglets born or other causes). On the contrary, at BD1, WT1 and BD2, the majority of subjects had a low content of cortisol, lower than 10 pg/mg. Therefore, to date, considering that we used a limited number of animals, we cannot provide an exhaustive comparative individual benchmark for animals under specific physiological, rearing and environmental conditions but, in the future, it will hopefully be possible to obtain that information for other stages of growth, sex (male, female, castrated) and rearing systems.

In conclusion, this research demonstrated that measurement of cortisol in swine hair could be regarded as a reliable method for furnishing information, otherwise very difficult to collect, on the animal welfare over a medium/long period of time. This technique demonstrated to be not invasive and the animal doesn't need to be restrained. Sampling can be easily carried out directly at the farm on many animals, and results can provide farmers with information on overall animal conditions over a medium/long period.

4.4 TABLES AND FIGURES

Season	Winter		Spring			Summer			Autumn	
Month and *ambient temperature (°C) (average min/max)*	Feb (2.1-9.8)	Mar (4.7-13.5)	Apr (7.6-18.4)	May (12.3-21.3)	Jun (16.4-26.5)	Jul (19.2-30.5)	Aug (17.5-28.4)	Sep (13.2-23.7)	Oct 9.4-17.6)	Nov (6.1-13.2)
Rearing	Farrowing crate	Single crate	Collective pen			Farrowing crate	Single crate	Collective pen		
and	Delivery / lactation	AI and pregnancy (114d)				Delivery / lactation	AI and pregnancy (114d)			
average *room temperature (min/max)*	22°C (21-23.5)	20°C (17.5-22.0)				27°C (24.5-29.0)	20°C (17.0-23.0)			
Sampling			PD1		BD1		WT1		PD2	BD2

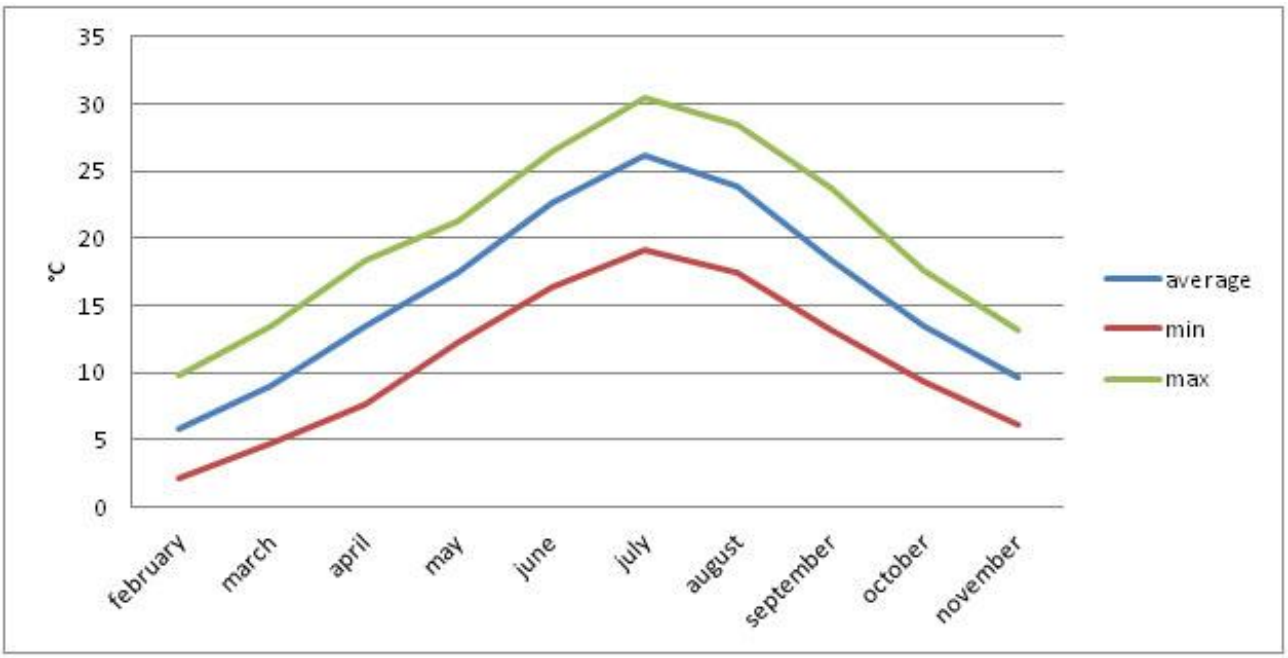

Figure 3. *Environmental temperature recorded in the vicinity of the stable in the year of the experiment*.

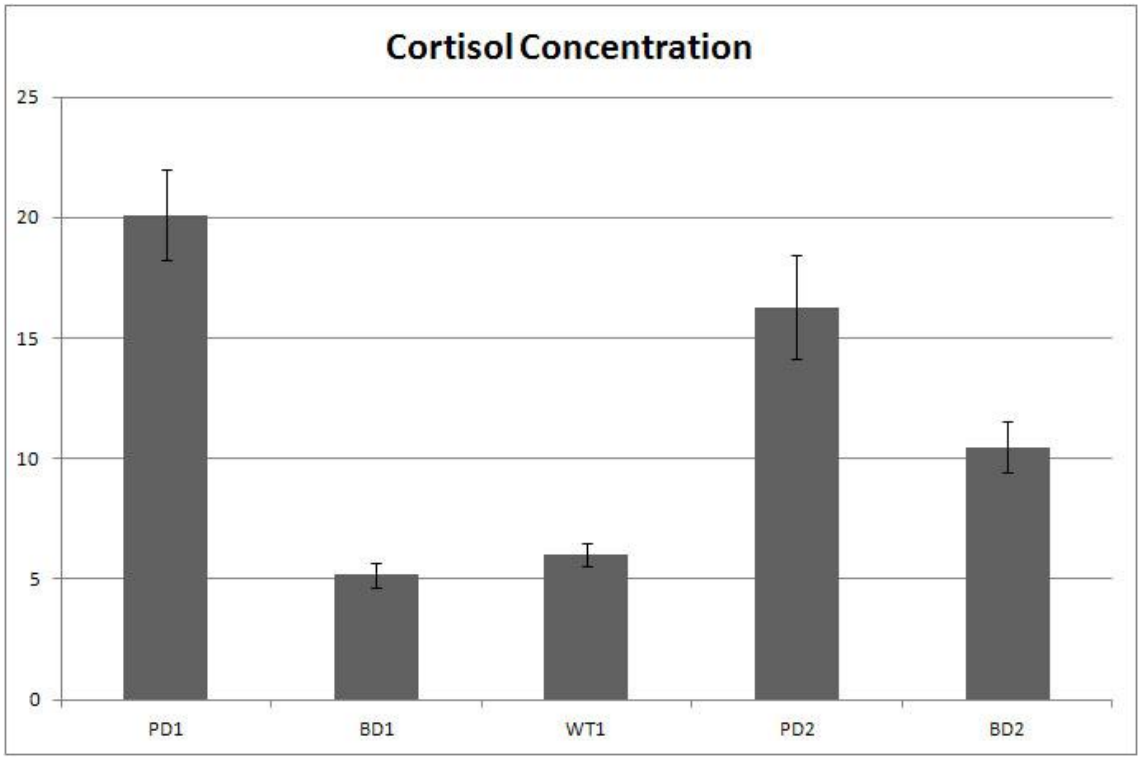

Figure 4. *Mean sow hair cortisol concentration*. PD: Pregnancy Diagnosis; BD: Before Delivery (5 days); WT: Weaning Time (28 days after delivery). PD1: April; BD1: June; WT1: July; PD2: September; BD2: November. (BD1, PD1, WT1: 30 sows; BD2, PD2: 25 sows). Different letters indicate statistically significant differences (P<0.001).

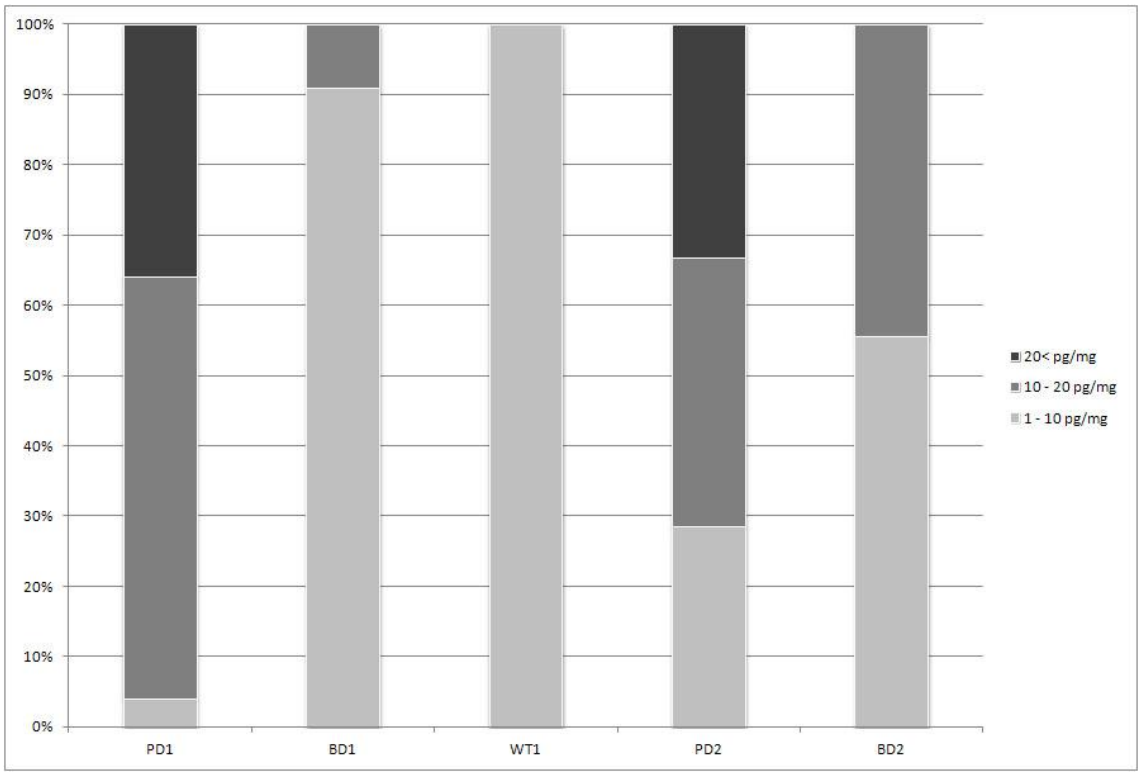

Figure 5. *Percentages of sows having different concentrations of cortisol in their hair*. PD: Pregnancy diagnosis; BD: Before delivery (5 days); WT: weaning time (28 days after delivery). PD1: April; BD1: June; WT1: July; PD2:September; BD2: November. The cortisol content category >20pg/mg is present only at PD1 and PD2. (BD1, PD1, WT1: 30 sows; BD2, PD2: 25 sows).

5. HAIR CORTICOSTERONE CONCENTRATION IN SPRAGUE-DAWLEY RATS AND ITS APPLICATION TO THE STUDY OF CHRONIC STRESS

5.1 Materials and Methods

5.1.1 Animals

The experimental protocols were approved by the Committee of Ethics of the Universitat Autònoma de Barcelona, following the "*Principles of laboratory animal care*" and were carried out in accordance to the European Communities Council Directive (86/609/EEC).

Male and female Sprague–Dawley rats obtained from the breeding center of the Universitat Autònoma de Barcelona were used. Animals were sixty days old at the beginning of the experiments and were assigned randomly to the experimental groups. Animals were housed individually (Experiments 1 and 2) or in pairs (Experiment 3) in polypropylene opaque wire-topped cages with a solid-bottom (Panlab S.L.U., Barcelona, Spain) containing wood shavings bedding (Lignocel, Harlan, Barcelona, Spain); standard temperature conditions (21 ± 1 °C) and a 12-12 h light/dark schedule (lights on at 08:00 h) were used. Food and water were available ad libitum. The animals were allowed to

acclimate to the housing conditions for at least one week prior to the beginning of the experimental treatments. Experiments were carried out in the morning.

5.1.2 Hair sampling

Rats typically have a docile and manageable temperament. Only few seconds of wrapping were necessary to collect a large amount of hair. Hair was shaved in the morning with an electric grooming clipper as close to the skin as possible. Approximately 50 cm^2 of old hair was removed from the ischiatic region of each rat. If a second sample of new hair was required by the experimental design, the sample was collected within the same area, being careful not to cut any old hair. Samples were placed into aluminum foil for protection, then into small plastic pouches, numbered, and kept at 4 °C until the time of assay.

5.1.3 Sample washing and extraction procedure

For hair corticosterone extraction we developed a modified washing protocol based on that reported by Davenport et al. (2006) in rhesus macaques and subsequently applied successfully in sows with slight modifications. In brief, for corticosterone extraction, hair samples were washed twice by a gentle rotation of 3 min with 15 ml of 2-propanol per ~200 mg of hair at RT; each wash was followed by centrifugation at 1500 × g (1 min, 4°C) and removal of the supernatant. Hair samples were put on aluminum foil inside an incubator at 37 °C for approximately 30 min to completely dry the samples. Dried hair was

frozen with liquid nitrogen and powdered in a mortar while it was frozen. 5 ml of methanol HPLC grade were added to 100 mg of powdered hair and incubated at room temperature with slow rotation for 21 h. After centrifugation at 10,000 × g (10 min, 4°C), 4 ml of supernatant were recovered in a glass tube and dried at 38 °C under a gentle stream of nitrogen gas. Dried extracts were reconstituted with 200 µl of 0.2 M sodium phosphate buffer, pH 7.6, and stored at -20 °C. On the day of the analysis, samples were centrifuged at 10,000 x g (10 min, 4 °C) and the supernatant was recovered and assayed. In the final validated protocol, 10 µl of reconstituted hair extract were used per tube.

5.1.4 Corticosterone radioimmunoassay and validation of hair corticosterone assay

Plasma and hair corticosterone levels were assayed by double antibody radioimmunoassay (RIA). Corticosterone RIA used 125I-corticosterone–carboximethyloxime–tyrosine–methylester (ICN, Barcelona, Spain) and synthetic corticosterone (Sigma, Barcelona, Spain) as the standard and an antibody raised in rabbits against corticosterone–carboximethyloxime-BSA that was kindly provided by Dr. G. Makara (Inst. Exp. Med., Budapest, Hungary). The characteristics of the antibody and the basic RIA procedure have been described previously (Zelena et al., 2003). All samples that were statistically compared were run in the same assay to avoid inter-assay variability.

To validate the described methods, the linearity, accuracy, precision and reproducibility of the assay were studied. The validation summary is shown in **Table 4**. Assay linearity was evaluated by assaying three different dilutions of three independent samples. A good parallelism between sample dilution and standard curve was found. Assay accuracy was tested by spiking five hair extracts with three known corticosterone concentrations and calculating the percentage of recovery ((measured corticosterone in spiked sample / measured corticosterone in non-spiked sample + corticosterone added)*100). Recovery was 97.9 ± 10.3 % (mean ± SD) indicating a good selectivity of the assay. Taken together, parallelism and selectivity results demonstrate the proportionality between hair corticosterone and the reference standard and the absence of significant matrix interferences, at least in the range of hair extracts assayed. Precision and reproducibility of the whole process (extraction + assay) were estimated by repeatedly extracting and assaying samples in the same assay (precision) or in two independent assays (reproducibility). The mean intra-assay and inter-assay CV were 4.3% and 12.7%. The lower corticosterone standard used in the assay was 6.25 pg/tube; using 8 mg of hair per tube, hair corticosterone can be assayed with reasonable accuracy and precision with a sensitivity of 0.78 pg/mg of hair.

5.1.5 Stressors

Two chronic stress procedures were used: chronic immobilization (IMOch) and chronic unpredictable stress (CUS). The chronic treatments lasted from experimental days one to nine. Control animals remained undisturbed.

5.1.5.1 Chronic Immobilization (IMO)

The first model consisted of daily exposure to 1 h of immobilization (IMO) at 09:30 h. IMO rats were stressed by taping their four limbs to metal mounts attached to a board (García et al, 2000). Head movements were restricted with two plastic pieces (7x6 cm) placed in each side of the head and the body was subjected to the board by means of a piece of plastic cloth (10 cm wide) attached with Velcro that surrounded the trunk.

5.1.5.2 Chronic Unpredictable Stress (CUS)

The CUS procedure consisted of exposing rats to different types of stressors: restraint, electric foot-shocks and forced swimming. For restraint, animals were placed into PVC cylindrical tubes measuring 6 cm diameter and 21.5-cm length for 30, 60 or 90 minutes. The rear top of the apparatus was closed by a cork letting the tail of the rat hang off. Several holes (0.5 cm in diameter) in the walls of the cylinder provided fresh air. For shock, rats received a six second shock (1.5 mA) each minute for 30, 60 or 90 minutes. Rats were put into individual clear Plexiglas boxes with a metal grid floor connected to a

shocker (Cibertec, Madrid, Spain). For the swimming condition, animals were placed in transparent cylindrical plastic tanks (height= 40 cm, internal diameter = 19 cm) containing water (25 °C) to a level of 24 cm, as described previously (Dal-Zotto et al. 2000). Animals remained in the tank for 20 min. The rats were then withdrawn from the water, returned to their home cages and exposed to swim for an additional 10 min period 1 h later. The CUS procedures involved changing the stressor and the time every day: day 1, 30 min restraint at 11:00 h; day 2, 30 min of shocks at 12:30 h; day 3, swim at 15:00 h; day 4, 60 min of shocks at 10:30 h; day 5, swim at 08:00 h; day 6, 60 min of restraint at 15:30 h; day 7, swim at 16:00 h; day 8, 90 min of restraint at 10:00 h; and day 9, 90 min of shocks at 14:30 h.

5.1.6 Experiment 1: Comparison of male and female rats

Sixteen rats (eight males, eight females) were housed individually and blood samples were taken under basal conditions at different time points on a single day (10:00 h; 13:00 h; 17:00 h; 20:00 h; 23:00 h; 10:00 h) to assess total plasma corticosterone circadian rhythm. Hair samples were collected at the end of the experiment.

5.1.7 Experiment 2: Effect of two chronic stress procedures on hair corticosterone

This experiment was conducted to assess if two extensively used chronic stress models increase hair corticosterone. Eighteen male rats housed individually were randomly assigned to the following three groups: control (n=6), IMOch (n=6) and CUS (n=6). On day one prior to starting the treatments, hair samples were taken from all animals. Body weight and food intake were measured several times throughout the experimental period. Hair was sampled at day 12 instead of on the day after the last stressor (day 10) because hair growth was not enough for removal. Animals were then sacrificed, and their adrenal gland and thymus were removed.

5.1.8 Experiment 3: Effect of chronic ACTH administration

The aim of this experiment was: (a) to demonstrate that hair corticosterone quantitatively reflects the magnitude of chronic corticosterone production; (b) to rule out a ceiling effect in hair accumulation that limits its validity to reflect actual corticosterone secretion under certain conditions.

To this end, we administered tetracosactide (Nuvacthén Depot 1 mg/ml; Defiante farmacêutica, Funchal, Portugal), a synthetic analogue of ACTH consisting of the first 24 amino acids of the naturally occurring hormone. This treatment maintains high levels of ACTH over the 24 h period, thus resulting in

a marked and prolonged activation of adrenocortical secretion (Riondel et al. 1987), while maintaining physiological levels of glucocorticoids. Twenty-four male rats housed in pairs were randomly assigned to three groups (eight x group) and received daily saline solution (control) or tetracosactide (20 μg/kg or 100 μg/kg) for 15 days, to allow appropriate hair growth. Injections were administered s.c. from days one to fifteen at 10:00 h. Hair samples were collected on day one (prior to starting the treatments) and again on day 16. Body weight and food intake were monitored throughout the experimental period. The values reported are the food consumption per day/rat, and the statistical unit was the cage. At the end of the experiment, animals were sacrificed and the adrenal gland removed.

5.1.9 Statistical analysis

Data were analyzed with the Statistical Program for Social Sciences (SPSS), version 17. A two way ANOVA with sex as the between-subjects factor (two levels), and time of day as the withinsubjects factor (six levels) was used for the study of the circadian rhythm of plasma corticosterone in Exp 1. When appropriate, further comparisons were performed. In other cases, a t-test was used to compare two independent means (i.e.: sex differences in hair corticosterone). The one-way ANOVA was followed by a post-hoc Student-Newman-Keuls (SNK) test when more than two independent means were compared (the criterion for significance was set at $p<0.05$ in the SNK test).

When variances were not homogenous between groups, logarithmic transformation of data were used. The area under the curve (AUC) for each animal was calculated from the circadian rhythm data with GraphPad Prism 5.

5.2 Results

5.2.1 Comparison of male and female rats

Figure 6 shows comparison of corticosterone concentration in male and female rats. The two-way ANOVA of the circadian pattern of plasma corticosterone revealed significant effects of sampling time ($F(5,70)= 4.6$, $p<0.001$), sex ($F(1,14)= 7.1$, $p=0.019$) and the interaction sampling time x sex ($F(5,70)= 3.9$, $p=0.021$). The decomposition of the interaction showed that plasma corticosterone levels were higher in females at 10:00 h. The t-test analysis revealed that the corticosterone AUC was significantly higher in females than males ($t(14)= 2.68$, $p=0.018$). However, no differences were found in the hair corticosterone concentration between sexes ($t(14)= -0.22$, $p=0.826$).

5.2.2 Effects of chronic stress

Table 5 shows the results for body weight gain, food intake and adrenal and thymus weights. Statistical analysis revealed significant differences between groups in body weight gain ($F(2,17)=26.2$, $p<0.001$) and food intake ($F(2,17)=14.3$, $p<0.001$). Post-hoc comparisons with the SNK test showed that CUS decreased body weight gain compared with controls, but the effect of

IMOch was more pronounced and differed from both control and CUS rats. Although CUS tended to decrease food intake, the effect only reached significance in IMOch compared to controls. Neither adrenal nor thymus weight were significantly affected by CUS or IMOch. Hair corticosterone concentrations before and after the chronic stress procedures are shown in **Figure 7**. We separately analyzed hair corticosterone before and after chronic stress because they represent completely different time domains for corticosterone accumulation: all of life is represented by the sample taken prior to the exposure to chronic stress and only the period of exposure to chronic stress is represented for samples taken after chronic stress. As expected, the one-way ANOVA revealed no group differences on day one samples (before chronic stress), whereas differences were significant on day 12 ($F(2,17)=10.2$, $p=0.002$). Further post-hoc comparisons showed that both IMOch and CUS significantly increased hair corticosterone concentration (approximately 80%) compared to controls ($p<0.05$), with no differences between both chronic stress paradigms.

5.2.3 Effects of chronic ACTH administration

Table 6 shows the results for body weight gain, food intake and adrenal weight. The one-way ANOVA revealed significant differences between groups in body weight gain ($F(2,23)= 70.4$, $p<0.001$), both absolute and relative adrenal weights ($F(2,23)=93.7$ and $F(2,23)=110.0$, respectively, $p<0.001$ in both cases)

and food intake ($F(2,11)= 6.3$, $p=0.019$). Post-hoc SNK comparisons showed that both doses of ACTH caused a statically significant reduction in body weight gain, with a significantly greater effect in the high dose group. The same pattern was observed for absolute and relative adrenal weights. ACTH treatment caused a reduction in food intake that was statistically significant only for the high dose.

Regarding hair corticosterone (**Figure 8**), the one-way ANOVA again did not reveal significant differences between groups on day one (before drug administration) but did on day 16 ($F(2,23)= 182.7$, $p<0.001$). A post-hoc SNK test revealed that both doses dramatically increased hair corticosterone concentration ($p<0.05$), with a much greater increase in the 100 µg/kg group. In the latter case, ACTH administration caused more than a 20-fold increase in hair corticosterone levels.

5.3 Discussion

The second study represent the first technical and biological validation of hair corticosterone measurement in Sprague-Dawley rats. Moreover, we have validated a specific washing protocol and extraction assay method that shows a good sensitivity. Additionally, for the first time, these results give tentative support to the hypothesis that hair corticosterone reflects plasma free corticosterone fraction and that it is a good marker of chronic HPA activation in typical chronic stress procedures lasting a few weeks.

The results of the first experiment, in male and female rats, showed the expected and well-characterized circadian pattern of plasma corticosterone with lower levels in the morning and the highest levels occurring around the onset of the dark cycle. As expected (Atkinson and Waddell, 1997; Hiroshige et al.,1973), females showed higher levels of corticosterone throughout the 24 h period than males. However, hair corticosterone concentration was similar in both sexes. As it has been reported that brain corticosterone concentration, which reflects free plasma corticosterone, is similar in male and female rats (Droste et al., 2008, 2009), our results support the hypothesis (Meyer and Novak, 2012; Russell et al., 2012) that hair glucocorticoids are a reflection of free rather than total circulating glucocorticoid levels. The present results are supported by prior studies in humans and other species failing to find sex differences in hair cortisol or higher levels in men under nonstressful conditions (Raul et al., 2004; Karlen et al., 2011; Dettenborn et al., 2012; Manenschijn et al., 2012; Stalder et al., 2012). Small and inconsistent differences have been reported in salivary cortisol, which reflects free cortisol, between men and women under resting conditions (King et al., 2000; Larsson et al., 2009; Lenderbogen et al., 2010). However, women consistently respond less to acute stressors (Kudielka et al., 2009). Nevertheless, alternative possibilities cannot be completely rule out, including sex differences in the rate of hair growth or hair corticosterone accumulation/degradation that may compensate for differences in circulating corticosterone.

Hair cortisol has attracted great interest as a means to evaluate chronic stress in humans and has potential as a fundamental tool in human stress research.

However, studies in experimental animals are needed to validate the procedure. To our knowledge, regarding laboratory animal field, there are only two important studies that aimed to characterize hair cortisol changes after chronic stress in non-human primates. Chronic stress was induced by moving animals from one facility to another or to a new social environment; in both cases, higher hair cortisol levels were observed 14 and 29 weeks after the chronic stress (Davenport et al., 2006; Fairbanks et al., 2011).

In this study, we also directly assessed the influence of two validated chronic stress models: chronic repeated IMO and CUS. By measuring not only hair corticosterone but also other well-known stress-related parameters (food intake, body weight gain, adrenal and thymus weight), we can better characterize the overall intensity of the stressors and compare the above parameters with the changes in hair corticosterone concentration. IMOch and CUS both significantly reduced body weight gain, and the former also reduced food intake, suggesting that a greater overall physiological impact of IMOch than CUS. However, both stressors increased hair corticosterone concentration to the same extent, demonstrating for the first time that hair corticosterone is appropriate to evaluate chronic stress in laboratory rodents. Although food intake and body weight gain are rather good markers of the severity of

stressors, a partial dissociation between different stress markers is not unlikely. Interestingly, in the present experiment, changes in relative adrenal weight of the adrenals and thymus were not significant. Thus, it appears that the measurement of hair corticosterone appropriately reflects chronic stress states lasting only nine days and that hair corticosterone is more sensitive than changes in adrenal or thymus weight.

Although the preceding results clearly demonstrated that hair corticosterone is a sensitive index of chronic stress, it is unclear the extent to which accumulation of corticosterone in hair saturates, thus diminishing its validity as a marker when using severe stressors and/or more prolonged periods of exposure. In fact, a saturation effect could explain the lack of differences between IMOch and CUS. To answer this question, we did an experiment injecting two doses of a long-acting ACTH preparation (tetracosactide), which slowly release ACTH and can stimulate the adrenal for hours after its administration. This procedure has been previously used in the literature and results in strong increases in adrenal weight (i.e., Stark et al., 1968; Alario et al., 1987; Giralt et al., 1987). Our results demonstrated dose-dependent reductions in body weight and food intake, as well as dose-dependent increases in adrenal weight and hair corticosterone. In fact, dramatic dose-dependent increases in hair corticosterone were observed. These data not only confirm that hair corticosterone reflects the magnitude of the chronic activation of the HPA axis but also showed no evidence for saturation of

corticosterone incorporation into the hair matrix. Therefore, it is clear that a ceiling effect in hair corticosterone accumulation under more natural conditions of chronic stress is unlikely.

In conclusion, the washing and extraction procedure used was validated for rats and could also be potentially applied to other laboratory animals, such as mice. The present data indicates that hair corticosterone concentration is similar in male and female rats and could reflect plasma free corticosterone levels. Hair corticosterone was elevated in response to chronic stress procedures and chronic ACTH administration, demonstrating that it provides a good direct index of HPA activity over long periods of time that better compares with indirect parameters, such as adrenal or thymus weight. This technique enables the use of rats to better characterize the impact of genetically or environmentally generated individual differences on the HPA axis and the impact on environmental conditions and chronic stressors, particularly those involving complex designs and stimuli.

5.4 TABLES AND FIGURES

1.1. Linearity		Dilutions	
Sample	1	1/2	1/4
1	100%	103%	104%
2	100%	97%	102%
3	100%	123%	137%
1.2. Recovery		Corticosterone added	
Sample	+50 pg	+100 pg	+200 pg
4	107%	97%	100%
5	110%	110%	93%
6	81%	100%	88%
7	80%	102%	98%
8	114%	89%	100%

Table 4. *Linearity and Recovery*: to assay linearity, three independent samples were diluted with a ratio of ½ and ¼. The percent of the expected value for each sample is shown. To assay recovery, three known concentrations of corticosterone were added (+50 pg; +100 pg and + 200 pg per tube) to five hair extracts. Percent recovery was calculated on the observed vs expected.

			Adrenal Weight		Thymus Weight	
	Body Weight Gain	Food Intake	Absolute	Relative	Absolute	Relative
Control	60.5±2.5	28.2±1.0	57.2±3.1	13.0±0.7	576.9±65.1	129.3±10.6
IMOch	12.2±5.7*	22.2±0.8*	53.8±2.5	13.6±0.6	450.3±52.7	115.9±16.0
CUS	29.9±5.4*$	26.2±0.5	59.6±3.1	15.3±0.7	451.8±33.1	117.2±10.7

Table 5. *Effects of two chronic stress procedures on body weight gain, food intake, and adrenal and thymus weight (Experiment 2)*: Body weight gain (g) and food intake (g/rat/day) were measured during the chronic stress period (between day 1 and 9). Adrenal and thymus weights (mg) were measured on day 12. Relative adrenal and thymus weights are shown as mg of tissue/100 g body weight. Means ± SEM (n=8 per group) are represented. Groups differences were analyzed by a one-way ANOVA followed by SNK post-hoc comparisons; * $p<0.05$ vs control group; $ $p<0.05$ between IMOch and CUS groups.

			Adrenal weight	
	Body Weight Gain	Food Intake	Absolute	Relative
Vehicle	84.3±4.3	47.0±1.7	41.5±2.3	12.0±0.5
20 µg/Kg	63.6±3.5 *	42.4±1.0	56.5±4.0 *	17.5±1.3 *
100 µg/Kg	12.8±5.2 *$	39.1±1.9*	113.9±5.0 *$	42.0±2.4 *$

Table 6. *Effects of two doses of chronic tetracosactide administration on body weight gain, food intake and adrenal weight (Experiment 3):* Body weight gain (g) (n=8 per group) and food intake (g/rat/day, n=4 per group) were measured during the chronic administration period (between day 1 and 15). Adrenal weight (mg) was measured on day 16. Relative adrenal weight is shown as mg of tissue/100 g body weight. In this experiment animals were housed in pairs so food consumption refers to the home cage intake and not to the individual animals. Means ± SEM are represented. Groups differences were analyzed by one-way ANOVA followed by SNK post-hoc comparisons; * $p<0.05$ vs vehicle group; $ $p<0.05$ vs the lower dose of the drug.

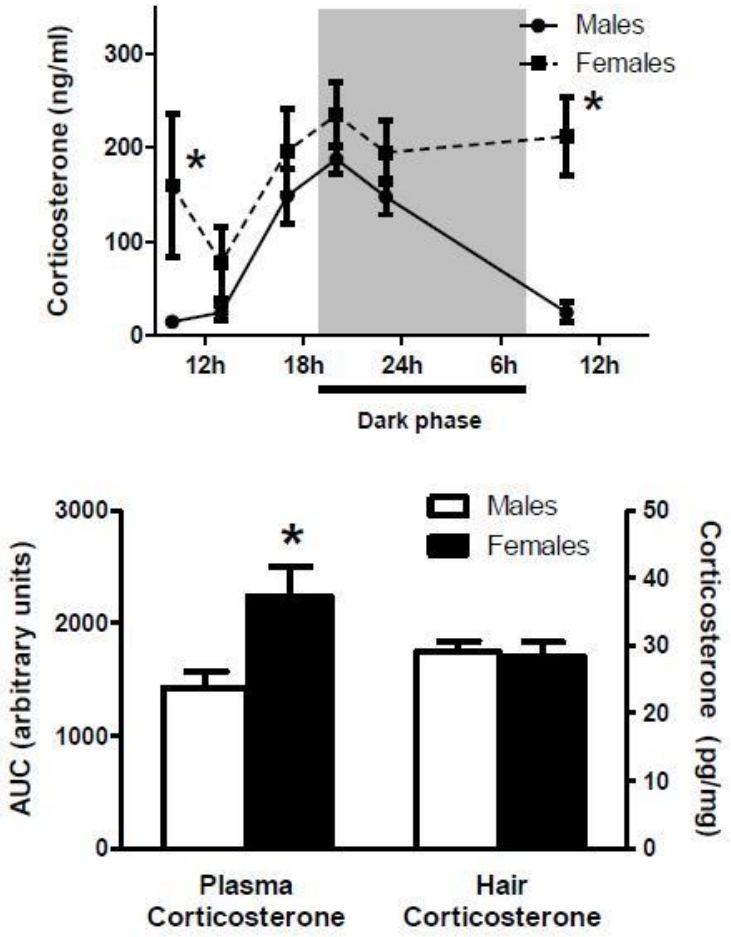

Figure 6. *Plasma and hair corticosterone levels in male and female rats*. The upper panel shows the circadian rhythm of total plasma corticosterone. Samples were taken at different time points during the lights on period (10:00 h, 13:00 h, and 17:00 h) and the nocturnal period (20:00 h and 23:00 h, marked in shadow). Means ± SEM (n=8 per group) are represented. Circadian rhythm data were analyzed by two-way ANOVA with repeated measures for the factor time. The area under the curve (AUC) of plasma corticosterone was calculated from the circadian rhythm data for each animal (left bottom panel). Hair corticosterone levels are shown in the right bottom panel; *p<0.05 between sexes.

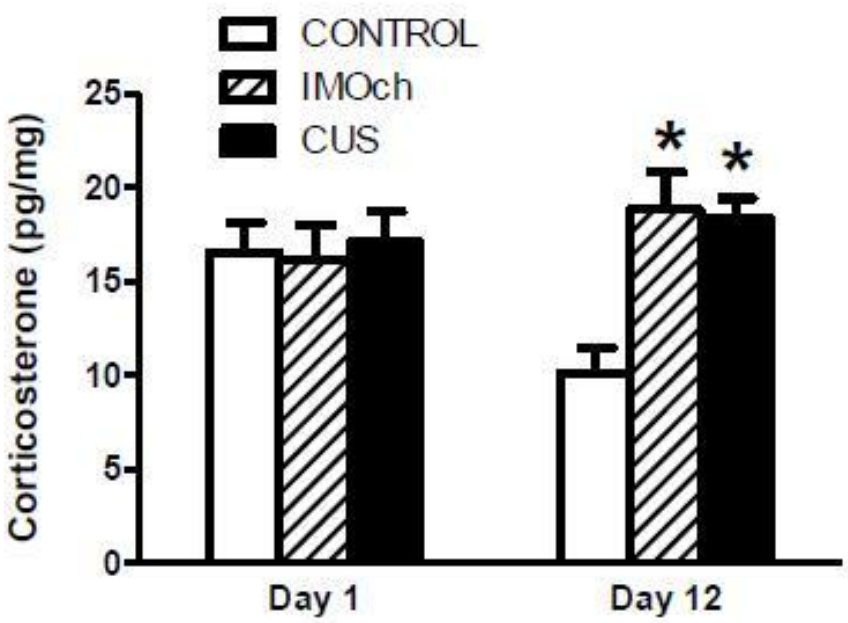

Figure 7. *Effects of two chronic stress procedures on hair corticosterone*. Chronic exposure to IMO (IMOch) or to various different stressors (CUS) started on day one and was completed on day nine. Hair samples were taken before the experiment (day 1) and at day 12. Means ± SEM (n=6 per group) are represented. For each day, groups were statistically compared with a one-way ANOVA followed by a SNK post-hoc test when appropriate; * $p<0.05$ vs. control group.

Figure 8. *Effects of chronic ACTH (tetracosactide) administration on hair corticosterone*. Vehicle or two doses of tetracosactide (20 µg/Kg and 100 µg/Kg) were injected from day one to fifteen. Hair samples were taken before (day 1) and after drug administration (day 16). Means ± SEM (n=8 per group) are represented. For each day, groups were statistically compared with a one-way ANOVA followed by a SNK post-hoc test when appropriate; * $p<0.05$ vs. control group; $ $p<0.05$ vs. 20µg/Kg group.

6. CONCLUSIONS

These studies support the utility of hair matrix as an important biomarker of HPA axis activity over long periods of time and of chronic stress in livestock and laboratory animals.

In commercial sows:

1. For the first time, it was developed and validated a new hair cortisol extraction procedure and we revealed the simplicity and the non-invasiveness of this technique.
2. Data on hair cortisol concentration changes in standard rearing conditions could be important references for further studies and may help to better characterize the impact of stressors on pigs welfare.
3. It was demonstrated the effect of the reproductive phase on hair cortisol concentration that might be correlated with different housing conditions during the breeding period.
4. The effect of seasonality with the lowest hair cortisol values recorded during the hot season may be related with one of several factors influencing the swine fertility during summer.

In Sprague-Dawley rats:

1. For the first time, our data demonstrated the validation of a specific washing protocol and corticosterone extraction assay method that has good sensitivity and that could be applied successfully in mice.
2. It was observed similar hair corticosterone concentration in male and female rats despite clearly higher total plasma corticosterone levels in females, suggesting that hair could actually reflect free fraction of corticosterone.
3. It was showed a markedly elevated hair corticosterone concentration in response to chronic stress manipulations and chronic ACTH administration, demonstrating that hair provides a good direct index of HPA activity over long periods of time that better compares with indirect parameters, such as adrenal or thymus weight.

7. LIMITATIONS AND FUTURE DIRECTIONS

Even though hair is able to reflect long-term of HPA axis activity and retrospective measurement of CORT secretion, there are still many gaps and lacks limiting its application, mostly in clinical settings. More studies are needed to better understand the mechanism of CORT incorporation in hair and how CORT concentration could vary along the hair shaft. To date, the degree to which hair CORT concentration is representing of the systemic bloodstream free fraction is uncertain.

The heterogeneity in the types of CORT extraction procedures and analysis are a challenge when trying to compare different studies. Mostly in animals, it is always essential to validate specie-specific washes and to establish a degree determining the negative effects of external contaminations.

Besides, it is important to remember that the RIA assay of hair samples has higher assay variability than the plasma ones, even if the analyses are performed by high skilled investigators with years of experience using this technique. This pitfall could be avoided using triplicates in the assay while investigating in the meantime the origin of this extra variability. However, the development of other procedures (i.e.: LC-MS/MS) are needed to overcome this issue within implying higher costs to process our samples.

As previously described, hair is not able to measure acute stress and still could not be considered as the gold standard biomarker of chronic stress. Hence, this matrix should be considered as complementary measurement of plasma/salivary CORT and not the replacement for such methods.

Finally, an elevated HPA axis activity does not always imply a stressful condition but could be correlated with physiological status (i.e.: physical exercises, body mass index, reproductive period). Stressed subjects could reveal bidirectional changes depending on stressor variable and some studies carried out in parallel with this research (unpublished) demonstrated a slower hair growing phase (anagen) in chronic stressed Sprague-Dawley rats that could limit the utility of this matrix.

However, it's believed that this new non-invasive technique will have a high impact on animal welfare, on the refinement principle and on laboratory animal science, mostly considering the extensive use of rats in bio medical research. Hair sampling has been demonstrated, by these studies, to be not stressful for the animal compared to blood collection and any other matrix is able to reflect the long-term accumulative impact of chronic stress exposure in animals. Subsequent research will need to investigate the use of hair as biomarker of stress in other experimental studies aiming at characterizing biological underpinning of mood disorders and depression in humans. Additionally, it could be interesting to validate this new technique in mice, which may require the use of procedures other than RIA to quantify corticosterone.

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