Identification of new physiological parameters for monitoring chronic stress in growing pigs: Hair cortisol and Chromogranin A

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Identification of new physiological parameters for monitoring chronic stress in growing pigs: Hair cortisol and Chromogranin A

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Certifiquen que la present tesi ha estat realitzada sota llur direcció, i considerant-la acabada, autoritzen la seva presentació per tal que sigui jutjada per la comissió corresponent.

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RESUM

Durant els darrers anys ha augmentat considerablement l'interès per desenvolupar tècniques no invasives que mesurin objectivament el benestar animal, i més concretament, l'estrès. La majoria d'estudis s'han dirigit a trobar indicadors efectius per avaluar l'estrès agut, i pocs s'han centrat en indicadors per mesurar l'estrès crònic. L'objectiu principal d'aquesta tesi és identificar i estudiar la viabilitat del cortisol en pèl i de la cromogranina A (CgA) com a indicadors d'estrès crònic en porcs d'engreix. Amb aquest propòsit es van realitzar quatre estudis englobats en dos experiments. El primer estudi tenia com a objectiu determinar la viabilitat del pèl com a matriu per detectar cortisol en l'espècie porcina. Per aquest motiu, es van analitzar mostres de pèl de 56 mascles sencers creuats [(Landrace x Duroc) x Pietrain] sotmesos a un procés de reorganització setmanal dels corrals. Els resultats obtinguts van mostrar una correlació positiva significativa de les concentracions de pèl en les diferents mostres preses al llarg del temps. A més, es van observar diferències en funció de la regió anatòmica mostrejada, amb majors concentracions de cortisol a la regió dorso-lumbar en comparació amb la regió cràneo-dorsal. Segons aquests resultats, el pèl és una matriu vàlida per detectar el cortisol en els porcs d'engreix. L'objectiu del segon estudi era analitzar l'ús del cortisol en pèl, la CgA en saliva i el factor de necrosis tumoral alfa (TNF-α) en sang com a possibles biomarcadors d'estrès crònic. Per aquest motiu es va sotmetre a 56 mascles sencers creuats [(Landrace x Larg white) x Pietrain] a diferents tractaments usant enriquiment ambiental (EE) i un compost natural d'herbes (HC) amb l'objectiu de reduir l'estrès. Els diferents tractaments van ser: a) porcs criats amb EE, b) porcs suplementats amb HC, c) porcs criats amb EE i suplementats amb HC (EEHC), i d) grup control (CG). Durant l'estudi, es van recollir mostres de pèl, saliva i sang a les 15, 20 i 24 setmanes de vida (T0, T1 i T2 respectivament). A T0, no es van observar diferències en cap indicador. En canvi, a T2, EE, HC i EEHC van presentar menors concentracions de CgA i cortisol en pèl en comparació amb el CG. A T1 es van observar diferències similars però només en el cas de CgA. A més, els valors de cortisol en pèl van correlacionar significativament amb els de CgA. Els resultats obtinguts en aquest experiment suggereixen que la concentració de CgA i el cortisol en pèl poden ser bons biomarcadors d'estrès crònic en porcs d'engreix. Al tercer estudi, es van analitzar possibles diferències comportamentals i de rendiment relacionades amb l' EE i l' HC. Per aquest motiu, es van pesar i es van avaluar les lesions de la pell de tots els porcs del segon estudi abans de començar l'experiment (15 setmanes d'edat) i a les 18, 20, 22 i 24 setmanes. A més, es van realitzar observacions setmanals de cada corral, i es van portar a terme tres tests de novetat a les 16, 19 i 23 setmanes d'edat. Al final de l'experiment (setmana 24), els animals del grup control van presentar un menor pes que els animals sotmesos a EE o HC. A més, els criats amb EE van presentar més comportament exploratori

i menys estereotípies i conductes redirigides. Per altra banda, els porcs suplementats amb HC van presentar menys interaccions socials i menys lesions a la pell. Els resultats obtinguts en aquest experiment suggereixen que tant l'EE com HC influeixen positivament sobre el creixement i el benestar dels porcs d'engreix. El quart estudi es va dur a terme amb els mateixos animals que l'estudi 2 i 3. L'objectiu d'aquest darrer estudi va ser avaluar la qualitat de la carn i la canal, així com l'acceptabilitat i la percepció dels consumidors respecte l' EE i HC. En general, no es van obtenir diferències de qualitat ni d'acceptabilitat per cap dels paràmetres analitzats. Per altra banda, l'aspecte més valorat pels consumidors va ser el sistema de producció, amb preferència per aquells sistemes que tenen com a objectiu incrementar el benestar, seguit per el tipus d'alimentació, amb preferència pels sistemes suplementats amb herbes naturals d'efectes relaxants. Per últim, el factor menys important va ser el preu. Tot i això, el preu va ser un factor important per un segment dels consumidors, amb una clara preferència pels preus baixos. Els resultats obtinguts suggereixen que les millores de benestar a granja són un factor apreciat per un important segment dels consumidors. En resum, els resultats dels dos experiments duts a terme suggereixen que el cortisol mesurat en pèl, i la CgA mesurada en saliva, poden esdevenir bons indicadors d'estrès crònic en porcs d'engreix.

ABSTRACT

During the last few decades, the interest to develop non-invasive techniques to objectively assess animal welfare and particularly stress response had increased considerably. Most of previous studies have focused in finding appropriate indicators to evaluate acute stress, but few attempted to find indicators to measure chronic stress. The main objective of this thesis is to identify and study the feasibility and reliability of hair cortisol and salivary Chromogranin A (CgA) as indicators of chronic stress in growing pigs. In this context, four studies were conducted encompassed in two different experiments. The first study aimed to determine the viability of hair as a matrix to detect cortisol in swine. For this purpose, hair samples from 56 crossbred [(Landrace x Duroc) x Pietrain] entire males subjected to weekly remixing were analysed. The results showed a significant positive correlation of hair cortisol concentration in the different samples taken throughout time. Furthermore, differences were observed in terms of the anatomical region sampled, presenting the dorso-lumbar region higher concentrations compared with the craneo-dorsal region According to these results, hair is a proper matrix to detect cortisol in growing pigs. The aim of the second study was to analyse the use of hair cortisol, salivary CgA and tumour necrosis factor alpha (TNF-α) in blood as potential biomarkers of chronic stress. For this reason 56 crossbreed entire males were subjected to different treatments using environmental enrichment (EE) and a compound of natural herbs (HC), and aiming to reduce stress. The treatments were: a) pigs reared in EE b) pigs supplemented with HC, c) pigs reared in EE and supplemented with HC (EEHC) and d) control group (CG). During the study, samples of hair, saliva and blood on 15, 20 and 24 weeks of age (T0, T1 and T2 respectively) were collected. At T0, no differences were observed in any indicator. However, at T2, salivary CgA and hair cortisol concentration were lower in EE, HC and EEHC compared to the CG. At T1, similar differences were observed, but only in salivary CgA concentration. In addition, the concentration of hair cortisol was significantly correlated with salivary CgA concentration. Results from this experiment suggest that salivary CgA and hair cortisol could be good biomarkers of chronic stress in growing pigs. In the third study, behaviour patterns and performance indicators were analysed in relation to EE and HC. For this reason, body weight and body lesions were recorded from all the pigs before starting the experiment (15 weeks old) and at 18, 20, 22 and 24 weeks. Moreover, weekly observations were performed from each pen, and three novel tests were carried out at 16, 19 and 23 weeks of age. At the end of the experiment (24 days), pigs from the control group showed a lower weight than pigs subjected to EE or HC. Furthermore, pigs reared with EE presented more exploratory behaviour and less stereotypies and redirected behaviour. On the other hand, pigs supplemented with HC presented less social interactions and less skin lesions. The results from this experiment suggest that

both EE and HC positively influenced animal welfare and performance of growing pigs. The fourth study was carried out with the same animals used in studies 2 and 3. The aim of this last study was to evaluate consumers' acceptability and preference, and meat and carcass quality of pork regarding the EE and HC. In general, there was no significant difference in quality or acceptability of any of the parameters analysed. On the other hand, the most important factor considered by consumers was the production system, with preferences for those systems aiming to increase the welfare, followed by the feeding system, with preference for systems supplemented with natural herbs with relaxing properties. Finally, the least important factor was the price. However, the price was an important factor for a segment of consumers with a clear preference for low prices. The present results suggest that improvements in animal welfare at farm level are appreciated by an important segment of consumers. In summary, results from both experiments suggest that cortisol measured in hair and CgA measured in saliva could be good chronic stress indicators in growing pigs.

“The fairest thing we can experience is the mysterious. It is the fundamental emotion which stands at the cradle of true science. He who knows it not, and can no longer wonder, no longer feel amazement, is as good as dead. We all had this priceless talent when we were young. But as time goes by, many of us lose it. The true scientist never loses the faculty of amazement. It is the essence of his being.”

-Hans Selye-

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SUMMARY OF ABBREVIATIONS

a*	tendency to red, objective measure of the colour with the Minolta Chromameter
ACTH	adrenocorticotropic hormone
AVP	arginine Vasopressin
b*	tendency to yellow, objective measure of the colour with the Minolta Chromameter
BE	barren environment
CgA	chromogranin A
CG	control group
CRH	corticotrophin releasing hormone
D	dorsal area of the neck
e.g.	for example
ECuLT	electrical conductivity measured in the *longissimus thoracis*
EE	environmental enrichment
eet	environmental enrichment treatment
EIA	enzyme immunoassays
EJC	subjective colour using a Japanese colour scale
ELISA	enzyme linked immunosorbent assay
Feeder type 1	precision feeder (Feeder type 1)
Feeder type 2	commercial electronic feeder system (Feeder type 2
G14	group size of 14 pigs
G21	group size of 21 pigs
G28	group size of 28 pigs
GRINLD	intramuscular fat content
HC	herbal compound
HC-	not supplemented with an herbal compound
HC+	supplemented with an herbal compound
hct	herbal compound treatment
HPA	hypothalamic pituitary adrenocortical
HPLC-MS	high performance liquid chromatography-mass-spectrometry
HS	housing system
Hz	Hertz
i.e.	id est ("that is")
L	loin part of dorso-lumbar region
L*	luminosity, objective measure of the colour with the Minolta Chromameter
LR3/4FOM	backfat
LT	*longissimus thoracis*
MFOM	muscle thicknesses
MLOIN	minimum fat and skin thickness over the *gluteus medius* muscle
NPPC	subjective marbling with pattern from National Pork Producers Council
ns	not significant
P, p	p-value
p.m.	post mortem
pHuLT	muscle pH at Longi+A1:C72+C57:C72ssimus thoracis 24 h p.m.
PNV	paraventricular nuclei
R	coefficient of correlation
R2	coefficient of determination
RIA	radio-immunoassays
RMSE	root mean square error
rpm	revolutions per minute
SAM	sympathetic adrenal medullary
sample 1	hair sample taken at 17 weeks of age
sample 2	hair sample taken at 21 weeks of age
SEM	standard error of the mean
T0	samples taken at 16 weeks of age
T1	samples taken at 20 weeks of age
T2	samples taken at 24 weeks of age
TNF-α	tumour necrosis factor-alpha
TR-IFMA	time-resolved immunofluorometric assay

General introduction

1. ANIMAL WELFARE

1.1. Animal welfare background

During the last decades, especially since the publication of Animal Machines by Ruth Harrison (1964), society has increased its interest for ethics and animal welfare issues (Broom, 2010; Fraser *et al.*, 1997; Sandøe and Simonsen, 1992), raising public awareness of how animals are treated in intensive animal production systems. In response to the public outcry, the British Government set up the Brambell commission, who wrote a report aiming to inquire the basic welfare requirements for the animals kept under intensive livestock production systems (Brambell, 1965). Brambell report launched animal welfare as a formal scientific discipline (Mench, 1998), and highlighted the needs for a new legislation related to the welfare of animals kept for food production. Thus, since scientific research on animal welfare started because of ethical concerns of the society, the assessment of animal welfare carried by scientists should be closely related with the ethical concerns socially constructed (Fraser *et al.*, 1997; Sandøe and Simonsen, 1992; Schmidt, 2011). Therefore, as stated by Fraser and Peerce (2004) "progress in animal ethics requires both philosophically informed science to provide an empirically grounded understanding of animals, and scientifically informed philosophy to explore the ethical implications that follow".

Nowadays, it is widely assumed that humans have ethical duties to animals (Sandøe *et al.*, 1997), and despite the existing high disparity of opinions regarding which are the morally acceptable levels of welfare, most people would not tolerate animals being kept in very poor welfare conditions, especially in systems involving pain (Broom, 1988). Furthermore there is the general perception that besides the well-being of the animals, increasing the welfare has also a positive impact in the food safety, quality and healthiness of consumers (Harper and Henson, 2001). Altogether, considering the abovementioned aspects, the demand for more welfare-friendly food products augmented considerably during the last years (Keeling, 2005; Napolitano *et al.*, 2010).

1.2. Animal welfare definition

Animal welfare is a multi-faced issue including scientific, ethical, economic and political dimensions (Lund *et al.*, 2006). The concepts of animal welfare have encountered difficulties to adequately deal with its multidimensional nature (Rushen, 1991). However, from a scientific point of view, animal welfare is considered as an intrinsic characteristic of the animals, and not something given to it

(Keeling *et al.*, 2011), and three main approaches have been followed to define and, consequently, to find methodologies to assess welfare levels (Carenzi and Verga, 2009; Duncan and Fraser, 1997).

The first approach establishes the link between animal welfare and an adequate biological function. An adequate biological function means that the animals are able to satisfy their biological needs (Hurnik and Lehman, 1988). From this point of view, Broom (1986) stated that welfare of an individual is "its state as regards its attempt to cope with its environment". When there is a failure to cope with the environment it can produce some problems affecting growth, reproduction, health and behaviour, leading to a poor welfare. From a biological perspective, it is important to emphasize that "to cope" means more than "to adapt", since adaptation does not necessarily mean good welfare (Broom, 2011).

A second approach is related with the mental welfare. As it is recognized in the Treaty of Amsterdam by the European Commission (1997), animals are "sentient beings". Positive emotional states are related with good welfare, while negative emotional states are assumed to reduce it (Murphy *et al.*, 2014). According to this approach, as long as the mental state is protected, animal welfare is safeguarded (Duncan and Petherick, 1991). In other words, it is more important how the animals feel than how the animals are (Veissier and Boissy, 2007). Thus, affect, emotions, feelings and suffering are considered as key elements in determining a good or a bad welfare (Broom, 2010), being the presence of positive affects even more relevant for the well-being of the animals than the absence of negative affects (Boissy *et al.*, 2007).

The third approach emphasizes the normal patterns of behaviour, or "natural-living" concerns, stating that animals should be allowed to live according to their natural attitudes and behaviour (Fraser *et al.*, 1997). Normal patterns of behaviour are usually associated with the behaviour performed by most subjects of the observed species in natural conditions. However, caution is needed in assuming an increased welfare when normal patterns of behaviour are expressed since some natural behaviours are clearly negative (such as fight). Moreover, it is fair to assume a certain modification of the behaviours related with the domestication process (Price, 1984), and consequently, the "normal behaviour" of wild animals and captive animals may be slightly different.

To take into account the abovementioned aspects, in 1979, the Farm Animal Welfare Council, defined five ideal states based on Brambell report (1965) which are a framework for the analysis of animal welfare: the so-called "Five Freedoms". These ideal states include absence of hunger and thirst, discomfort, pain, disease or injury, fear and distress, and freedom to express normal behaviour. They also considered that any animal kept by man must be protected from unnecessary

suffering (FAWC 1992). The Five Freedoms have been included into many animal welfare legislations and codes of recommendations all over the world (McCulloch, 2013). However, despite the five freedoms are seen as a general guideline for non-specialists and represent a useful preliminary guideline in science, they are not precise enough to be used as basis for welfare assessment (Broom, 2011).

More recently, the OIE (World Organization for Animal Health, Office international des Epizooties in French) incorporated animal welfare as a priority, and some guiding principles were included in the Terrestrial animal health code in 2004. Table 1 contents the 11 General principles for the welfare of animals in livestock production systems recognised in the latest version ((25th edition, May 2016).

Table 1: General principles for the welfare of animals in livestock production systems (OIE, 2016) recognized in the article 7.1.4. of the chapter 7.1. *Introduction to the recommendations for animal welfare*, of the section 7. *Animal welfare*, in the 25th edition of the Terrestrial animal health code.

1) Genetic selection should always take into account the health and welfare of animals.
2) Animals chosen for introduction into new environments should be suited to the local climate and able to adapt to local diseases, parasites and nutrition.
3) The physical environment, including the substrate (walking surface, resting surface, etc.), should be suited to the species so as to minimise risk of injury and transmission of diseases or parasites to animals.
4) The physical environment should allow comfortable resting, safe and comfortable movement including normal postural changes, and the opportunity to perform types of natural behaviour that animals are motivated to perform.
5) Social grouping of animals should be managed to allow positive social behaviour and minimise injury, distress and chronic fear.
6) For housed animals, air quality, temperature and humidity should support good animal health and not be aversive. Where extreme conditions occur, animals should not be prevented from using their natural methods of thermo-regulation.
7) Animals should have access to sufficient feed and water, suited to the animals' age and needs, to maintain normal health and productivity and to prevent prolonged hunger, thirst, malnutrition or dehydration.
8) Diseases and parasites should be prevented and controlled as much as possible through good management practices. Animals with serious health problems should be isolated and treated promptly or killed humanely if treatment is not feasible or recovery is unlikely.
9) Where painful procedures cannot be avoided, the resulting pain should be managed to the extent that available methods allow.
10) The handling of animals should foster a positive relationship between humans and animals and should not cause injury, panic, lasting fear or avoidable stress.
11) Owners and handlers should have sufficient skill and knowledge to ensure that animals are treated in accordance with these principles.

2. STRESS

2.1. Concept of stress

As stated by the "Five Freedoms", a potential indicator of animal welfare is the absence of stress. Yet, as it has been said, it is difficult to attain welfare under negative stress conditions (Veissier and Boissy, 2007), which usually present damaging effects. The concept of stress was first introduced by Hans Selye (1936) who described a syndrome that appears after a "noxious agent" and produces a non-specific response to damage as such. Considering that the syndrome as a whole seems to represent a generalized effort of the organism to adapt itself to new conditions, he termed it "general adaptation syndrome." Since that moment, the concept of stress has been subjected to an intense debate, and new ideas and definitions have aroused. Furthermore, the highly extended use of this word by society together with the intuitive feelings of each person often adds more complexity to the definition (Moberg, 2000). However, although the term "stress" is full of ambiguities (McEwen, 2000), it is often defined as a threat to the homeostasis of the organism (Chrousos and Gold, 1992), even when the threat is not real, but it is perceived as such (Chrousos, 2009).

Animals do have the ability to adapt to new situations by physiological, morphological and behavioural modifications (Möstl and Palme, 2002). However, unpredictable situations or situations exceeding their capacity to adapt may difficult the homeostasis maintenance. Once stress is perceived, it activates a broad range of complex physiological, behavioural and neurological changes in order to re-establish the homeostasis (Chrousos, 2009).

The term "homeostasis" was used for the first time by Cannon (1932), based on the concept of "milieu interieur" proposed by Claude Bernard in 1854. Homeostasis can be considered as all the mechanisms of the organism employed to maintain its constancy, or, in other words: a self-regulation of the inner-equilibrium. However, according to different authors, the definition of stress as a threat to the homeostasis is nearly meaningless, suggesting that almost all the activities of an organism represent a threat to the homeostasis (Koolhaas *et al.*, 2011). Consequently, the term allostasis (Sterling and Eyer, 1988), which means stability through changes, has been proposed to better clarify the concept of stress (McEwen, 2005). Allostasis refers to the physiological mechanisms that maintain the homeostasis (Romero *et al.*, 2009). According to allostasis, an unusual physiological parameter is likely produced as a response to some predictions, not as a consequence of failing to adapt. Thus, the emotional brain, by means of previous experiences, feelings, memories and re-evaluation of needs, can influence in the anticipation of physiological requirements (Korte *et al.*,

2007). Therefore, as suggested by McEwen (2000), allostasis is the process that keeps the organism alive and functioning while homeostasis should be reserved only for the parameters that are essentially maintained to survive.

Another important aspect still under debate is the existence or not of "good stress". Selye *et al.* (1976) suggested the use of the terms "eustress", referred to agreeable or healthy stress, and the term "distress" referred to the disagreeable or pathogenic stress. However, the term "stress" is commonly employed in the negative sense of "distress" (McEwen, 2000). Therefore, some scientific such as Broom (2011) suggested that good stress does not exist, and the definition of stress should be based only in the adverse effects or distress, and thus, on the general perception of "stress".

Despite the negative effects produced by stress, it is not inherently bad (Moberg, 2000). Every animal feels stress from time to time. Actually, stress can be live-saving because it is a signal of disturbance, an alarm telling to the body that something impaired. Thus, the paradox of stress lies in the simultaneity of its adaptive nature and its possible maladaptive consequences (Korte *et al.*, 2007).

In this work, stress will be defined as the state produced by uncontrollable and/or unpredictable situations from the environment overloading the natural regulatory capacity of an organism, and representing a threat to the homeostasis, even if these situations are not real but are perceived as such, producing adverse physiological and behavioural consequences.

2.2. Stress response

There are enormous individual differences in front of a potentially stressful situation with the same intensity. These differences are mainly related with the genetic inheritance, experience, constitutional factors and in how the subject perceive and interpret the situation (McEwen, 1998; McEwen and Stellar, 1993; Schneiderman *et al.*, 2005). Thus, for the same stressor, different individuals may react in a completely different way. Consequently, the interaction between the stressor and the organism can lead to three different situations (Chrousos, 2009):

1) The organism is capable to overcome the stressor and returns to the basal homeostasis.

2) The organism not only is capable to overcome the stressor but also to improve the homeostasis capacity because of the experience gained.

3) The adaptive response is inappropriate, and the organism fails to cope with the challenging threats, producing a biological response in an effort to recover the homeostasis.

The stress response begins when the central nervous system perceives a potential threat to the homeostasis of the organism, whether or not the threat is real. Once the threat is perceived, it develops a complex biological response of defence formed by behavioural changes, multiple hormones interacting with each other and the immune response (Moberg, 2000). The objective of this response is to increase the oxygenation and nutrition to the brain, heart and skeletal muscles, preparing the body for an emergency situation and for a quick reaction. Furthermore, in order to increase the chance of survival, there is a decreased pain perception and the organism diverts resources from non-vital physiological processes such as the maintenance of the immunity, reproduction, metabolism or growth, which are not critical in an emergency situation (Manteuffel, 2002). Most of these processes are controlled by neurotransmitters and hormones.

In general, all the animals try to face the stressor with the minimum energetic expense. However, when the challenge is high, the individual will attempt to cope regardless of cost (Moberg, 2000). For example, these two different stress scenarios could be compared: the first one consists in a prey facing a predator, while the other consists in a lion trying to avoid the sun in a very hot day in the savannah. In most cases, the demand and willingness to spend energy will be higher for the prey trying to escape from the predator compared with the energy required by a lion to go under a shadow.

The first and most biologically economical defence response consists in behavioural changes (Moberg, 2000). The animal may try to avoid the stressor by means of a predominantly behavioural response (Barnett and Hemsworth, 1990). Using the previous example, the lion will go to rest in a shadow (biological stress response) to avoid the extremely hot temperatures (stressor) of the savannah.

Usually, Behavioural modifications are not enough to cope with the stressors, appearing a physiological response, which involves an efficient and highly conserved set of interlocking systems aiming to maintain physiologic integrity even in the most demanding of circumstances (Ulrich-Lai and Herman, 2009). Along that lines, the sympathetic adrenal medullary (SAM) system and the hypothalamic pituitary adrenocortical (HPA) axis are generally considered the key players of the stress response (Koolhaas *et al.*, 2011).

Finally, in prolonged reactions, other neuroendocrine factors such as growth hormone, prolactin, and nerve growth factor are released to regulate the immune system during the stress response (Marketon and Glaser, 2008).

3. ANIMAL WELFARE IMPROVEMENT

During the last decades, the increasing demand for more animal friendly systems (Keeling, 2005) together with the benefits obtained when the welfare is taken into consideration (Harper and Henson, 2001), pushed science to investigate different measures aiming to enhance the welfare of the animals (Millman *et al.*, 2004).

In the present work, two strategies have been used in order to reduce the stress and increase on farm pig's welfare. These strategies consisted in the provision of environmental enrichment and the use of natural tranquilizers.

3.1. Environmental enrichment

Environmental enrichment is a concept which describes the changes produced in the environment of captive animals with the aim to increase the benefits of the inhabitants (Shepherdson, 1994). Enriched environments enhance the wellbeing of the animals by increasing behavioural diversity and normal behaviour patterns (understanding normal as those behaviours observed in wild animals), resulting in an overall augmentation of "desirable" behaviours, and a reduction of the frequency of abnormal or "undesirable" behaviours (Chamove, 1989; Young, 2003). Furthermore, enrichment has stress-reducing effects, it facilitates the ability to cope with challenging situations (Young, 2003), and it may improves cognitive performance (Grimberg-Henrici *et al.*, 2016). Lower levels of glucocorticoids have been reported in the animals reared in enriched conditions compared with the groups reared in barren conditions (Belz *et al.*, 2003; De Jong *et al.*, 2000; Roy *et al.*, 2001). The utilization of environmental enrichment is therefore considered as a way to enhance the welfare of the animals (EFSA, 2007), although other factors such as space allowance, group size or flooring conditions should not be overlooked (Averós *et al.*, 2010).

According to Van de Weerd and Day (2009), adequate environmental enrichment in growing pigs should meet four criteria to succeed:

- It should increase species-specific behaviour: thwarting the species-specific behaviours may produce disturbance in the physiology and behaviour of the animals (Wiepkema and Koolhaas, 1993). Pigs reared in barren conditions present limitations to express their foraging species-specific behaviour, which is considered a need for pig's welfare (Studnitz *et al.*, 2007). Limitations to express this exploratory behaviour can lead to frustration, producing behavioural and physiological responses in an attempt to satisfy innate needs (Van de Weerd and Day, 2009), and sometimes, redirecting the exploratory behaviour to other pen mates (Beattie *et al.*, 2000, 1995; De Jong *et al.*, 1998).
- It should maintain or improve levels of health: materials used should be appropriate or their effects should be more beneficial than detrimental, without compromising the health or safety of the animals. As an example, although it has been suggested that straw bedding may have a negative impact on the health of pigs increasing some pathogens and dust, most of the effects produced by straw are positive for the health of the animals if provided in accordance with the environmental conditions of each farm. Evidently, human health and safety must be also safeguarded by environmental enrichment (Newberry, 1995).
- It should improve the economics of the production system: the provision of enrichment material may reduce the aggressions and increase the performance of the animals, increasing the economic benefits (Van de Weerd and Day, 2009). However, the choice of the enrichment material is often based more on economic or health-related factors, than on the requirements of the animals (Van De Weerd *et al.*, 2003). This type of enrichment materials not primarily based on the animals' needs, may become meaningless to pigs after a certain time. Thus, although economic reasons are important, they should not be the primary goal of the environmental enrichment (Newberry, 1995).
- Finally, it should be practical to employ: if enrichment material is not practical, its implementation will be hampered. Farm operators have a preference for environmental enrichment that is suspended compared with enrichment offered at floor level (Scott *et al.*, 2009), especially in systems with partly or fully slated flooring (Van de Weerd and Day, 2009).

Barren environmental conditions of most modern pig husbandries are associated with signs of chronic stress (Beattie *et al.*, 2000; De Jonge *et al.*, 1996; Munsterhjelm *et al.*, 2010). Such is the importance of environmental enrichment for the wellbeing of pigs, that it is regulated in the European Union by means of the EU Directive 2008/120/EC. The present Council Directive requires that "pigs must have permanent access to a sufficient quantity of material to enable proper investigation and manipulation activities, such as straw, hay, wood, sawdust, mushroom compost,

peat or a mixture of such, which does not compromise the health of the animals". This statement was written by the first time in the EU Directive 2001/93/EC and entered into force in 2003. However, the current wording of the Directive leaves too much room for interpretation, and sometimes, the materials supplied are not the adequate or are not properly used (Bracke, 2006).

The effectiveness of the environmental enrichment lies mostly in the material used, the quantity and the location where it is placed, although "novelty" is also an important factor to consider (Van De Weerd *et al.*, 2003). The preferred materials that best stimulate exploratory behaviour are "complex", "changeable", "destructible", "manipulable", and "contain sparsely distributed edible parts" (Studnitz *et al.*, 2007). Thus, the enrichment material that offers more welfare benefits are straw and compound objects, followed by rubber, rope, wood, roughage and substrates, whilst metal objects offers few advantages (Bracke *et al.*, 2006).

3.2. Natural Tranquilizers- Herbal compounds

Another described strategy to reduce stress is by means of plants with sedative and tranquilizer properties such as Valeriana (*Valeriana officinalis)* and Maypop (*Passiflora incarnata)* (Murphy *et al.*, 2010; Peeters *et al.*, 2004; Soulimani *et al.*, 1997). The mechanism involved in the sedative and anxiolytic properties of *Valeriana officinalis* seems to be mediated by the interaction of Valerian Acid with the γ-Amino butyric acid receptors type A ($GABA_A$). The stimulation of $GABA_A$, opens the permeability of chloride channels, producing neural inhibition (Khom *et al.*, 2007; Murphy *et al.*, 2010). Moreover, different bioactive compounds have been detected in *Passiflora incarnata* such as flavonoids, maltol, cynogenic glycosides and indole alkaloids, without a consensus regarding the most important one for the sedative properties. Nevertheless, flavonoids, which are beyond the most studied components, have a similar effect than the abovementioned Valerian Acid, increasing the membrane permeability by means of the modulation of GABAA (For a review see: Miroddi *et al.*, 2013).

4. ANIMAL WELFARE AND STRESS ASSESSMENT

Animal welfare is a multidimensional concept which cannot be assessed by a single measure (Broom, 1988; Dawkins, 2003; Mason and Mendl, 1993), rather a great variety of parameters are needed for the overall welfare assessment (Fraser, 1995), including resource-based and animal-based measures (Smulders *et al.*, 2006). The different criteria used for assessing welfare are mainly based on evidence of change, although changes per se are not an indicator of impoverished or enhanced welfare (Barnett and Hemsworth, 1990). Because of its complexity, some problems arise when trying to assess welfare. One is that different measures may not co-vary, and thus, different measures may suggest contradictory results. Another problem is that some measures may rise up or decrease in completely different situations. As an example, adrenaline may rise up because of an acute stress situations, but also in a pleasurable experience (Mason and Mendl, 1993).

Stress and welfare have been usually considered as opposite versions of a common process. However, the link between indices of stress and poor welfare has not been widely discussed (Veissier and Boissy, 2007). Stress is usually assessed by means of animal based measures. However, because of the different effects produced by acute and chronic stress, different indicators may be appropriate in the evaluation of different stress responses (Broom, 2011). As an example, heart-rate or plasma cortisol will be more appropriate for assessing acute stress, while some behavioural measures and hair cortisol will be more valid for chronic stress assessment.

4.1. Resource-based measures

Resource-based measures include basically all the measures not related directly with the animals. They mainly consist in environment-based and management-based measures, and include all those measures related with the stockpersons (such as stockman competence and handling skills), the environment (such as housing conditions, type of floor, cleanliness, water and food facilities, type of bedding, thermal comfort, ventilation, stocking density, etc.) and factors that affect the welfare of the animal (such as appropriate genetic, health plans, etc.) (Butterworth, 2009; Whay *et al.*, 2003).

4.2. Animal based measures

Animal-based measures are all of those parameters directly related with the animals (Bracke, 2007). Parameters related with the biological function are usually easy to measure. On the contrary, most of parameters related with the mental welfare are poorly defined, impossible to measure directly, and difficult to measure indirectly (Duncan, 2005). Thus, biological function can be measured more objectively than mental welfare, which is mainly assessed throw subjective measures because indicators of mental welfare usually include a more subjective component of evaluation and interpretation (Lawrence, 2008).

Animal-based measures can be divided in four major categories: Performance records, behavioural measures, physiological measures and clinical parameters (Smulders *et al.*, 2006).

5. BEHAVIOUR AND STRESS RESPONSE

In daily live, behaviour may be interrupted and disarranged by a great variety of events evoking uncertainty. Examples of these events are the presence of a predator or the absence of food in an expected location (Wiepkema and Koolhaas, 1993). The modification of these behaviours can be produced by a primary response of the organism, but also as a consequence of the physiological changes caused in the organism such as the release of catecholamines and glucocorticoids (Moberg, 2000). These behavioural changes are adaptive, and considered as a natural response in front of a challenge; however, problem arises when housing conditions are such that organisms cannot solve the conflicts. This is the case when retreat or escape from the conflict situation is impossible, or when normal behaviours (for instance, foraging behaviour) cannot be performed or are limitedly performed (Wiepkema and Koolhaas, 1993).

Behaviour clearly indicates poor welfare when it involves physical suffering, and it is the most used parameter to assess pain on farm (Viñuela-Fernández *et al.*, 2007). However, the link between behaviour, chronic stress and animal welfare is not easily quantifiable. Opposite behavioural responses are found in stressful contexts depending on species, age of the animal, and probably also on its genetic and experiential background as well as the stressful situation per se (Veissier and Boissy, 2007). Some behavioural measures previously related with chronic stress include a reduced reproductive behaviour, increased abnormal behaviour, reduced exploratory behaviour, increased vigilance behaviour and hiding, reduced behavioural complexity, increased aggression, increased fearfulness, increased intensity, duration and frequency of startle responses, increased “fight or

flight" responses, increased freezing behaviour and decreased latency to freeze (Cook *et al.*, 2000; Morgan and Tromborg, 2007; Olsson *et al.*, 1999).

In general, abnormal behaviour can be considered as an indicator of poor welfare (Gonyou, 1994). Abnormal behaviour has been defined as "a persistent, undesirable action shown by a minority of the population which is not due to any obvious neurological lesion and which is not confined to the situation that originally elicited it" (Duncan and Dawkins, 1983). Some abnormal redirected behaviours such as tail biting, ear biting or ear chewing and belly noising, may appear when exploratory behaviour cannot be fulfilled and the animals redirect their active behaviour to the pen-mates (Van De Weerd *et al.*, 2003; Zwicker *et al.*, 2013). Other important abnormal behaviours closely related with thwarting the exploratory behaviour are the stereotypies (Mason and Latham, 2004). Stereotypies are repetitive, invariable and functionless behaviours closely related with stress and poor animal welfare (Mason *et al.*, 2007). Bar-biting in pigs, tong-rolling in cows or crib-biting in horses are some examples of stereotypic behaviour.

Behavioural differences have also been reported in front of novelty of stimuli in animals, with presumably different levels of stress. Novel object tests are commonly used to assess fear and anxiety (Dalmau *et al.*, 2009). A latency to touch a novel object, less time spent investigating the stimulus, and a higher diversity of behaviours during the novel test have been related with higher levels of anxiety or fear (Murphy *et al.*, 2014; Wemelsfelder *et al.*, 2000).

6. ENDOCRINOLOGY OF THE STRESS RESPONSE

During the last years, there has been a substantial growth in the use of non-invasive methods to quantify hormone production through the measurement of excreted hormones or hormone levels in different biological samples (Buchanan and Goldsmith, 2004).

The brain is the responsible of interpreting and responding to potential stressors. It is also a target for the action of stress hormones, particularly glucocorticoids (McEwen, 2000). The recognition of the stressor takes place in different areas of the brain depending on the nature of the stressor. An immediate physical stressor often is recognized in the amygdala while a delayed or abstract stressor is perceived in the cerebral cortex (Reser, 2016). Both amygdala and cerebral cortex together with other areas of the brain involved in the stress response have extensive connections to the hypothalamus (Bremner, 1999). One of the main functions of the hypothalamus is to link the nervous system and the endocrine system.

The adrenal glands have a key-role in hormonal reactions to stress as they are involved both in the sympathetic adrenal medullary (SAM) system and hypothalamus pituitary adrenal (HPA) axis (Möstl and Palme, 2002). The main hormones released by the SAM system are catecholamines, released immediately after the stress perception, whereas the main hormones produced by the HPA axis are glucocorticoids, with a longer lasting response (Manteuffel, 2002; Matteri *et al.*, 2000; Möstl and Palme, 2002). Catecholamines are released from the adrenal medulla, while glucocorticoids are released from the adrenal cortex.

Both SAM system and HPA axis are involved in the regulation of energy fluxes in order to cope with the stressors and maintain the homeostasis. Catecholamines and glucocorticoids are able to produce energetic metabolites either from energy storage tissues (the autonomic nervous system mobilizes fat from adipose tissues and glycogen from liver) or by gluconeogenesis (enhanced by glucocorticoids) (Mormède *et al.*, 2007).

6.1. Autonomic nervous system and the sympathetic adrenal medullary (SAM) system.

The autonomic nervous system is remote from voluntary control and is the responsible to keep body functions stable (Gabella, 2001). Moreover, it provides the most immediate response to stress exposure through its sympathetic and parasympathetic arms (Ulrich-Lai and Herman, 2009), both with antagonistic functions. Thus, the sympathetic nervous system stimulates the internal organs, increases the heart rate and blood pressure, dilates pupils, speeds breathing and diverts blood from digestive system. SAM system is part of the sympathetic nervous system, and it is associated with feelings of alertness and action proneness (Lundberg and Frankenhaeuser, 1980), preparing the animal for a situation described by Cannon (1915) as “fight or flight”.

SAM system consists of some preganglionic fibres of the splenic nerve travelling from the spinal cord to the medulla of the adrenal gland, where their nerve endings form synapses onto the parenchyma cells of the adrenal medulla (named chromaffin cells) (Aunis, 1998). When stress is perceived, acetylcholine is released from the nerve endings, and it binds to the adrenal chromaffin cells, producing the release of the catecholamines adrenaline and noradrenaline (also named epinephrine and norepinephrine) (Gabella, 2001). Among other actions, these hormones stimulate gluconeogenesis and lipolysis, and mobilize energy stores preparing the body for the “fight or flight” response. Furthermore, catecholamines stimulate the release of glucocorticoids and their precursors, preparing the body for a longer stress response (Matteri *et al.*, 2000).

Catecholamines are difficult to measure because of their rapid secretion and brief half-life (Dimsdale and Moss, 1980). Furthermore, their rapid degradation do not reflect short term changes in the activity of the SAM system (Schwab *et al.*, 1992). However, sympathetic stimulation increases salivary protein secretion, while parasympathetic stimulation increases salivary flow rate. Therefore, different salivary proteins such as α-amylase, immunoglobulin A (IgA) and Chromogranin A (CgA) have been related with stress and sympathetic stimulation (Obayashi, 2013).

6.2. Hypothalamus pituitary adrenal (HPA) axis

The activation of the HPA axis is a primary physiological response to stress in mammals. The HPA axis is related with both acute and chronic stress, and involves a neuroendocrine cascade culminating in the synthesis and secretion of glucocorticoids (Herman *et al.*, 2012). Cortisol is the primary glucocorticoid in most mammals and fishes, whereas in rodent and birds, it is corticosterone (Mormède *et al.*, 2007).

Glucocorticoids are cholesterol-derived steroids synthesised in the adrenal cortex under the control of the adrenocorticotropic hormone (ACTH) (Figure 1). ACTH is produced in the pituitary gland, and its release into the systemic circulation is triggered by several neuroendocrine hormones, being the corticotrophin releasing hormone (CRH) the most important (Charmandari *et al.*, 2005). Arginine Vasopressin (AVP) has very little ACTH secretogogue activity, but possesses the ability to potentiate CRH-induced ACTH secretion (Matteri *et al.*, 2000). Both CRH and AVP are synthesized in the paraventricular nuclei (PNV) of the hypothalamus and are released into the hypophyseal portal circulation. PNV receive a multiplicity of signals coming from other hypothalamic nuclei, from the brain stem, from the limbic system and from the subfornical organ. Altogether, it explains the sensitivity of the HPA to a wide range of stimuli from both internal and external origins (Mormède *et al.*, 2007). Thus, when a stressful stimulus is perceived there is an increase of the CRH and AVP secretion, which produce an increased ACTH in the systemic circulation, increasing glucocorticoids' synthesis and secretion. Cortisol release in blood is not immediate. It takes place after a few minutes from the beginning of the stimuli, and it is maintained for about one hour after the end of the stressor (Mormède *et al.*, 2007).

The primary action of glucocorticoids is the redistribution and provision of energy to cope with the stressors (Herman *et al.*, 2012). Thus, gluconeogenesis is enhanced by stimulating the liver to convert fat and protein to intermediate metabolites ultimately converted to glucose (Matteri *et al.*, 2000), and potentiates the activity of catecholamines. Furthermore, in order to save energy, glucocorticoids

decrease less immediately essential activities such as feeding, digestion, growth or reproduction (Sorrells and Sapolsky, 2007).

ACTH and glucocorticoids release are clearly rhythmic. In diurnal animals, basal concentrations of cortisol in blood are generally higher in the morning reaching a nadir in the evening (Ruis *et al.*, 1997), although a peak in the afternoon has also been described in some studies (Evans *et al.*, 1988). Furthermore, a blunted circadian rhythm and an elevated circadian trough have been related with chronic stress situations (De Jong *et al.*, 2000).

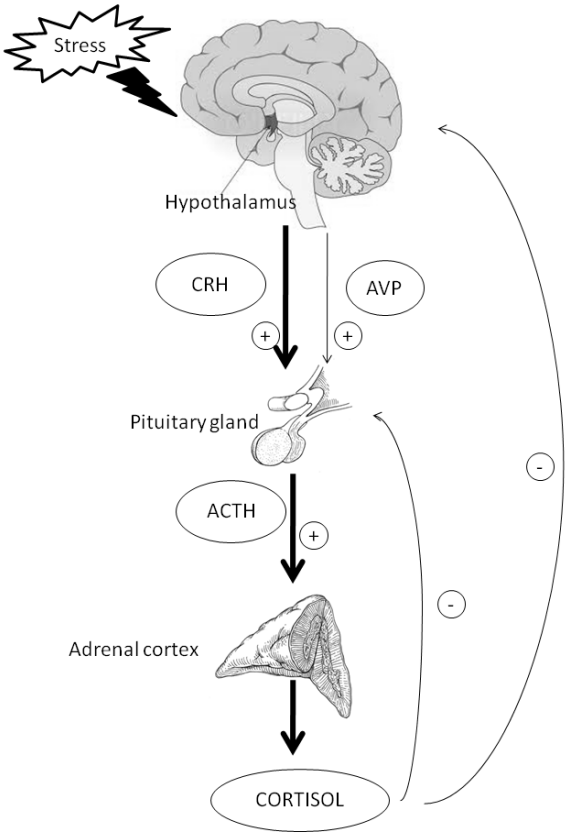

Figure 1: Schematic representation of the main components and hormones of the hypothalamic–pituitary–adrenal (HPA) axis where CRH is corticotrophin releasing hormone, AVP is arginin vasopressin and ACTH is adenocorticotropic hormone.

HPA axis is regulated by a complex negative feedback (Herman *et al.*, 2012) in order to return glucocorticoids and the rest of the hormones involved in HPA axis to the basal levels (Manteuffel, 2002). Excessive levels of glucocorticoids, are beneficial or harmless for a few days, however, negative consequences may appear when the levels are high during prolonged periods of time (Herman, 2009; Sapolsky *et al.*, 2000). Furthermore, a chronic stress exposure can induce physical

changes in the structure and function of some brain regions involved in the control of HPA axis (Ulrich-Lai and Herman, 2009) and may produce changes in the HPA feedback regulation, increasing the excitability of both HPA axis and SAM system. Moreover, prolonged exposure to glucocorticoids has been related with loss of neurons and an inhibition of neuronal regeneration (Bremner, 1999; McEwen, 2000).

7. PHYSIOLOGICAL INDICATORS OF STRESS

7.1. Chromogranin A

Chromogranin A (CgA) is the major member of the granins (or chromogranins), which are acidic secretory glycoproteins secreted in the chromaffin granules (or chromaffin secretory vesicles) found in many endocrine and neuroendocrine cells (Domínguez *et al.*, 2012; Hendry *et al.*, 1995). Chromogranins are characterized by highly hydrophilic and acidic sequences of amino acids. Among their main functions, chromogranins act as precursors of multifunctional hormones, facilitate the release and secretion of different granule-like structures, and help in the storage of catecholamines and ATP (Domínguez *et al.*, 2012).

CgA represents about 40% of the soluble proteins in the chromaffin granules (Simon *et al.*, 1988). It is stored and co-released with catecholamines to the blood from the vesicles of the adrenal medulla and the sympathetic nerve endings (Helle *et al.*, 2007; O'Connor and Frigon, 1984). Furthermore, CgA can be produced in the submandibular glands of humans from where it is secreted into saliva (Saruta *et al.*, 2005). Apparently, no relation exists between salivary and blood CgA since no correlation has been reported so far between them (Den *et al.*, 2007).

CgA release takes place in response of the sympathetic nerve stimuli (Kanno *et al.*, 2000). Salivary CgA peak after a few minutes, but lasts for up to one hour after the elimination of the stress (Obayashi, 2013), presenting a higher stability than catecholamines. Thus, it has been proposed to be a good indicator of SAM system activity, and hence, of acute stress (Escribano *et al.*, 2013; Gallina *et al.*, 2011; Kanai *et al.*, 2008; Nakane *et al.*, 1998).

Since CgA is taken from saliva, it can be assessed noninvasively, it is a pain-free method, and it produces minimum stress to the animals. However, CgA presented a circadian rhythm pattern in human saliva but not in blood (Den *et al.*, 2007). Although, this fluctuation was not observed in dogs (Kanai *et al.*, 2008) and pigs (Escribano *et al.*, 2014), circadian rhythm should be considered in those

species were it has been observed, and caution is needed for other species were no information is available.

The relation between physical or physiological acute stress and salivary CgA has been previously reported in humans (e.g. Kanamaru *et al.*, 2006; Mitsuhata *et al.*, 2012) and in pigs (Escribano *et al.*, 2015; Ott *et al.*, 2014). However, before this work, the use of salivary CgA has not been investigated as a possible chronic stress indicator.

7.2. Cortisol

Cortisol is one of the most measured steroid hormones since it is released as a consequence of the HPA axis activation (Nicolson, 2007; Thienpont, 1998). It can be assessed in several biological samples, the most common are plasma, saliva, urine, faeces, milk and hair (Mormède *et al.*, 2007; Möstl and Palme, 2002).

7.2.1. Cortisol in plasma and saliva

Plasma is the most widely used sample to assess glucocorticoids. Cortisol can be detected in plasma a few minutes after the exposure to the stressor, and levels are increased up to one hour after the exposition (Merlot *et al.*, 2011). Blood sampling is an invasive procedure that involves the capture and restraint of the animal, which is by itself stressful (Creel, 2001). For this reason, samples should be taken trying to reduce the elapsed time after restraining the animals.

Most of circulating cortisol is bound to proteins (principally albumina and cortiscosteroid binding globulin), and only about 10% of total cortisol is present in the blood as the free form, which is the biologically active. However, whether free cortisol is a better functional measure of HPA axis activity than total cortisol is not clear (Cook *et al.*, 1996; Mormède *et al.*, 2007). In pigs, the main places used to collect blood are the superficial veins of the ears, tail, vena cava anterior and jugular vein (Mormède *et al.*, 2007).

Saliva cortisol can be detected less than 5 minutes after its appearance in blood (Vining *et al.*, 1983), and presents a good correlation with plasma levels (Cook *et al.*, 1996; Merlot *et al.*, 2011; Vincent and Michell, 1992). In most mammals, including pigs, the concentration of salivary cortisol is about 10-15% of cortisol concentration in plasma (Ekkel *et al.*, 1996; Merlot *et al.*, 2011).

Saliva can be sampled easily and noninvasively without the necessity to restraint the animals, being less stressful and painful than blood collection (Mormède *et al.*, 2007). For this reasons, it is suggested to be a better option than blood collection for evaluating HPA axis response (Hellhammer *et al.*, 2009). In pigs the samples are usually obtained allowing the animals to chew on cotton buds until they are soaked with saliva (Smulders *et al.*, 2006).

Both saliva and plasma cortisol levels are used as indicators of acute exposure to stress. However, an important aspect to consider in both blood and saliva cortisol determinations is the circadian rhythm of its release, especially when different samples are needed in different days. In diurnal animals such as pigs, basal concentrations of cortisol in blood are generally higher in the morning reaching a nadir in the evening (Janssens *et al.*, 1995; Ruis *et al.*, 1997), although some studies also found a peak in the afternoon (Evans *et al.*, 1988).

7.2.2. Cortisol in urine, faeces and milk

Faeces and urine can be useful to measure corticosteroid released in the hours previous to sampling, since excretion products accumulate during several hours into the body. Thus, they can be used to evaluate sub-acute HPA axis activity (Hayssen *et al.*, 2002), but they are not suitable to evaluate acute stress due to the delay between stress and excretion (Merlot *et al.*, 2011). Urine and faeces can be obtained noninvasively. However, collection may be difficult, especially in wild animals (Stephen and Ledger, 2006). Steroids in faecal and urine samples are highly prone to degradation probably because of the high levels of bacteria present in the samples (Buchanan and Goldsmith, 2004). Both excretory products are less sensitive than plasma or saliva (Mormède *et al.*, 2007).

Milk cortisol and plasma cortisol concentration are in equilibrium since the free cortisol moves between them by simple diffusion, presenting a high correlation (Verkerk *et al.*, 1998). However, it can only be assessed in lactating animals (Möstl and Palme, 2002).

7.2.3. Cortisol in Hair

Hair samples have been widely used over the last 20 years in toxicology, forensic science, doping control and clinical diagnostics for the detection of environmental agents, toxins, drugs, hormones, alcohol, etc. because it provides a retrospective calendar of chronic and/or past exposure to those substances (Boumba *et al.*, 2006; Kirschbaum *et al.*, 2009; Koren *et al.*, 2002).

The assessment of hair cortisol can be considered an objective way to determine chronic stress because it is a measure of long term HPA axis activity (Gow *et al.*, 2010; Ullmann *et al.*, 2016; Van Uum *et al.*, 2008). Furthermore, hair cortisol has been previously correlated with cortisol in faeces (Accorsi *et al.*, 2008), saliva (Bennett and Hayssen, 2010; Davenport *et al.*, 2006), and urine (Sauvé *et al.*, 2007).

Cirimele *et al.* (2000) were the first to detect glucocorticoids in human hair although the first to detect endogenous cortisol and cortisone were Raul *et al.* (2004). Since then, hair has been used as an indicator of HPA axis activity in multitude of species including humans (D'Anna-Hernandez *et al.*, 2011; Dettenborn *et al.*, 2010; Nakane *et al.*, 1998; Sauvé *et al.*, 2007; Skoluda *et al.*, 2012; Ullmann *et al.*, 2016; Vliegenthart *et al.*, 2016), non-human primates: Vervet monkeys (Fairbanks *et al.*, 2011; Laudenslager *et al.*, 2012, 2011), chimpanzees (Carlitz *et al.*, 2016, 2015; Salaberger *et al.*, 2016; Yamanashi *et al.*, 2013), rhesus macaques (Davenport *et al.*, 2006; Dettmer *et al.*, 2012), other wild animals (Bechshøft *et al.*, 2013; Koren *et al.*, 2002), and domestic and farm animals: in cats and dogs (Accorsi *et al.*, 2008; Bennett and Hayssen, 2010; Siniscalchi *et al.*, 2013) dairy cattle (Comin *et al.*, 2011; Tallo-Parra *et al.*, 2015) or horse foals (Comin *et al.*, 2011). Before the publication of this thesis, up to the knowledge of the authors, hair cortisol in pigs had been studied twice: Martelli *et al.* (2014) in transgenic pigs and their relatives, and Bacci *et al.* (2014) in sows.

Although the mechanisms to explain how cortisol has access to hair are still not completely understood, the most commonly suggested hypothesis (Pragst and Balikova, 2006; Stalder and Kirschbaum, 2012) is based on the complex multi-compartment model proposed by Henderson (Henderson, 1993), used to explain drug incorporation into hair. This model suggests that cortisol may be incorporated into hair medulla primarily via passive diffusion from blood capillaries into the growing hair cells. Besides incorporation from blood, substances can be incorporated from deep skin compartments during hair shaft formation, although the most important alternative sources are diffusion from sweat and sebum secretions to the outer cuticle (Pragst and Balikova, 2006). Furthermore, glucocorticoids are locally produced in the hair follicle in a functional equivalent to

HPA axis (Ito *et al.*, 2005), and recently, Keckeis *et al.* (2012) suggested that only small amounts of systemic cortisol are incorporated into hair.

The main advantages of hair cortisol analyses compared with other biological samples are: (1) it is not affected by momentary stress (Yang *et al.*, 1998), (2) it is a non-invasive and a painless method, (3) sampling procedure is not complex and can be carried by a non-professional, and (4) once sampled, it can be stored at room temperature (Gow *et al.*, 2010). Furthermore 10 and 20 mg of hair have been suggested to be sufficient for the analysis (Van Uum *et al.*, 2008). However, in those animals with little hair, or small animals e.g. piglets, it may be difficult to collect the amount required for the analysis.

One of the most remarkable inconveniences of hair cortisol analysis is the requirement for a validation in each species analysed because of the differences in cortisol rhythm, secretions and stress response (Buchanan and Goldsmith, 2004). Moreover, many different factors such as hair growth rates, gender, age, hair colour, environmental exposure and others could influence the final concentration (Gow *et al.*, 2010). This high inter-individual variability contrasts with the high intra-individual stability in hair cortisol concentrations (Stalder *et al.*, 2012).

Different studies have used different hair cortisol analysis techniques such as enzyme linked immunosorbent assay (ELISA), simply enzyme immunoassay (EIA), radio-immunoassays (RIA) and high performance liquid chromatography-mass-spectrometry (HPLC-MS) (Gow *et al.*, 2010).

8. IMMUNOLOGY AND STRESS

The relation between stress and immune system has been widely studied during the last years. However, there are still many gaps to completely understand how the immune system responds to stressors because of the complexity of both the stress response and the host immunity system (Blecha, 2000). Although the influence of stress on the immune system is variable and could be individual specific (Chandrashekara *et al.*, 2007), stress has been linked to immune suppression and with a higher incidence of certain disease (Glaser *et al.*, 1987). Some of the described effects of stress over the immune system are a reduction of natural killer cell activity, reduction in the number of lymphocytes, decreased ratio of helper to suppressor T cells, decreased antibody production, reactivation of latent viruses and modulation of cytokine production (Marketon and Glaser, 2008).

Most of the effects of the stress response in the immune system are controlled by glucocorticoid and catecholamines (Padgett and Glaser, 2003). Those hormones can deregulate immune function producing an immune activation in acute and short-lived stress situations, but an immune suppression when the stress is chronic and persistent (McEwen, 2000). More concretely, the activation of the stress response is produced by catecholamines, which are potentiated by glucocorticoids (Sorrells and Sapolsky, 2007), while systematically exposure to glucocorticoids produce the suppression of the cellular immune response because of its effects on the target tissues (Elenkov, 2004). Thus, glucocorticoids have an effect in both stimulating and suppressing the immune response. The main immunological effects of stress hormones consists in inhibiting the traffic of neutrophils, macrophages, antigen-presenting cells, natural killer cells and lymphocytes T and B, impair the function of macrophages, natural killers and lymphocytes, and modify the production and regulation of cytokines (Padgett and Glaser, 2003).

Glucocorticoids produce a suppression of Th1-cellular immunity axis and a shift toward Th2-mediated humoral immunity producing changes in the cytokines derived from lymphocytes and monocytes. Th1 cells primarily secrete IFN-γ, IL- 2, and TNF-α, which are considered the major pro-inflammatory cytokines, while Th2 cells secrete primarily IL-4, IL-6, IL-10, and IL-13 which are the major anti-inflammatory cytokines (Elenkov, 2008; Elenkov and Chrousos, 2002; Paik *et al.*, 2000). Thus, a reduction of pro-inflammatory cytokines and an increase of anti-inflammatory cytokines are expected in chronic stress situations (Kang and Fox, 2001). However enhanced levels of pro-inflammatory cytokines have also been reported in chronic stress situations (e.g. Maes *et al.*, 1998) and in acute stress situations (e.g. Himmerich *et al.*, 2013). These inconsistencies are suggested to be related with the type (psychological vs. physiological vs. physical), duration (chronic vs. acute) and intensity of stressors, detection methods, and individual differences such as genetics or age (Salak-Johnson and McGlone, 2007; Tian *et al.*, 2014).

9. MEAT AND CARCASS QUALITY AND STRESS

Stress may produce physiological and biochemical alterations in pigs, which will affect the perimortem muscle metabolism and, thereby, the quality of the meat (Cassens *et al.*, 1975). When animals are slaughtered, after bleeding, the anaerobic muscular metabolism continues in the absence of oxygen and nutrients. The anaerobic muscular metabolism produces a decrease of the muscular glycogen levels in an attempt to provide the energy required to maintain the integrity of

the cells. Consequently, acid lactic is produced and protons and lactate are accumulated in the muscle, causing an acidification (Guyton and Hall, 1996; Terlouw, 2005).

When catecholamines are released as a consequence of acute stress situations, the rate of glycolysis is increased, augmenting the concentration of lactate and protons (Støier *et al.*, 2001), and the breakdown of glycogen in the muscle is stimulated (Merlot *et al.*, 2011). If pH declines too fast, it produces the denaturation of muscle proteins because of the association of low pH and relatively high muscle temperature, producing a pale, soft and exudative (PSE) meat (Briskey, 1964). On the other hand, the depletion of muscle glycogen produced by stress hormones may produce a lower reduction of pH than expected, leading to dark, firm, and dry (DFD) meat (Klont *et al.*, 2001). Usually, acute stress previous to slaughter is related with PSE meat, while a long lasting stress response is related with DFD meat (Adzitey and Nurul, 2011). However, other conditions such as the genotype are considered an important source of variation in ultimate pH (Fernandez and Tornberg, 1991).

Both cortisol and catecholamines have also been related with growth and performance. Cortisol has a proteolytic and lipolytic activity in peripheral tissues and anabolic activity (including gluconeogenesis and protein synthesis) in the liver (McMahon *et al.*, 1988; Mormède *et al.*, 2007). Furthermore, cortisol increase blood glucose and raise insulin concentration (Sapolsky *et al.*, 2000). The combination of increased cortisol and insulin leads to the storage of energy as fat in the adipose tissue (Mormède *et al.*, 2007). Thus, a chronic activation of the stress system would be expected to increase visceral adiposity and decrease lean body mass (Tsigos and Chrousos, 2002), which may affect the carcass quality of the animals subjected to stress. On the other hand, catecholamines increase the use of energy stores by means of catabolic actions stimulating the lipolysis and glycogenolysis, although an anabolic effect on muscle protein metabolism has also been reported (Navegantes *et al.*, 2002). As a consequence, it may produce a reduction of the carcass fat content, and increase the yield of the muscle (Navegantes *et al.*, 2002; Smulders *et al.*, 2006). Furthermore, glycogen in muscle is capable of binding water. Thus, the depletion of glycogen can be related with higher water losses in the muscle (Fernandez and Tornberg, 1991).

10. SOCIAL IMPLICATIONS OF ANIMAL WELFARE

Different intrinsic characteristics of pork meat such as colour, taste, texture, and odour, and some extrinsic characteristics such as price, food safety, origin and information on animal production may influence consumer behaviour (García-Torres *et al.*, 2016; Grunert *et al.*, 2004). Furthermore, these different attributes are not perceived with the same importance by different consumers. Thus, not all consumers have the same beliefs, attitudes and behaviours, towards the same product (Verbeke *et al.*, 1999). Although animal welfare appears to be among the most important factors to consider for some pork meat consumers (Meuwissen *et al.*, 2007), this is not always translated into real consumers' choices, even if they show a certain willingness to pay for welfare. (Harper and Henson, 2001). Access to information regarding the welfare issues and trust in the information provided by the labels have been suggested to be among the most important factors for those consumers aiming to purchase animal-friendly products (Toma *et al.*, 2012). Along that lines, most of the consumers indicate a lack of information about production systems and market transparency (Gracia *et al.*, 2009). However, most of them are not willing to know some details of how animals are treated, but would like to have a more transparent, enforceable, and traceable information with a labelling system for animal welfare products (Frewer *et al.*, 2005). On the other hand, there is the general perception that the interest of the industry in animal welfare is merely economic (Kendall *et al.*, 2006). In this context, a labelling system which guarantees an optimal animal welfare in farming practices is seen as one of the best alternatives.

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Objectives

OBJECTIVES

1- To evaluate the feasibility of hair as a matrix to determine cortisol in pigs (chapter 1 and 2).

2- To evaluate the feasibility and reliability of hair cortisol and salivary chromogranin A as an indicator of chronic stress in pigs using two strategies to reduce stress (environmental enrichment and herbal compound supplementation) (chapter 1 and 2).

3- To contrast the effects of two strategies to reduce stress (environmental enrichment and herbal compound supplementation) on hair cortisol and chromogranin A with other welfare indicators such as behaviour and performance (chapter 3).

4- To evaluate the effects of two strategies to reduce stress (environmental enrichment and herbal compound supplementation) on meat and carcass quality and consumers' acceptability and purchasing intention. (chapter 4).

Chapter 1

Analysis of cortisol in hair samples as an indicator of stress in pigs

Based on a paper submitted to Journal of Veterinary Behavior, accepted with minor revision

ABSTRACT

Detection of cortisol is one of the most widely used methods to assess stress in animals because it provides information of the hypothalamic–pituitary–adrenal axis activity. The most common biological samples are plasma, saliva, urine, faeces, milk and hair. Hair cortisol analysis could be a good non-invasive procedure to detect chronic stress since cortisol is incorporated and stored inside growing hair. The aim of this study was to determine whether cortisol could be detected in pig hair and could serve as a proper chronic stress indicator. Hair samples from two regions (cranio-dorsal (D) and dorso-lumbar (L)) of 56 crossbred entire male pigs were taken at 17, and 21 weeks of age. The pigs were subjected to a weekly remixing procedure. The mean cortisol level for the hair samples was 19.30 ± 0.63 pg/mg (range 6.4-43.88). Hair from second sampled day had higher cortisol values ($P=0.002$) than hair from first sampled day. Furthermore, L region had higher values than D region at every age measurement ($P<0.001$). Significant positive correlations were found between first sampled day and second sampled day in both region D ($r=0.442$ $P=0.019$) and region L ($r=0.523$ $P<0.001$). There were also correlations between both regions for first day ($r=0.595$ $P<0.001$) and for second day ($r=0.523$ $P<0.001$). Thus, cortisol could be detected in pig hair. However, some methodological improvements and constraints were detected, and further studies are required before recommending its use as a chronic stress indicator.

1. INTRODUCTION

The stress response, a concept first introduced by Hans Seyle (1936), begins when the central nervous system perceives a potential threat to the homeostasis of the organism. As a consequence, it produces the activation of the hypothalamic–pituitary–adrenal HPA) axis (Chrousos, 2009; Mellor *et al.*, 2000; Stewart *et al.*, 2007). HPA axis response is related to both acute and chronic stress and glucocorticoids are the final outputs of the HPA axis, being cortisol the main glucocorticoid in most mammals (Matteri *et al.*, 2000). Glucocorticoids can be measured in several biological samples such as plasma, saliva, urine, faeces, milk and hair (Mormède *et al.*, 2007). Blood, saliva and milk cortisol levels are used to determine acute stress because they provide a short term view of the HPA axis response (Verkerk *et al.*, 1998; Vining *et al.*, 1983), but in urine and faeces cortisol accumulates during several hours (Hayssen *et al.,* 2002). During the last years, the interest in non-invasive techniques to assess animal welfare, and specially stress response, has increased because of ethical and logistic reasons. Furthermore, the interest in hair samples is increasing because it provides a retrospective calendar of chronic and/or past exposure. Glucocorticoids in hair were first detected by Cirimele *et al.* (2000) in humans, and by Koren *et al.* (2002) in animals. Since then, multiple studies on the use of hair in different species have been carried out, most of them related with chronic stress detection.

The most commonly suggested hypothesis to explain how cortisol has access to the hair is based on the complex multi-compartment model that has been used to explain drug incorporation in hair (Cone, 1996; Henderson, 1993). This model suggests passive diffusion mainly from blood, but also from sweat, sebum secretions and external sources as primary incorporation routes. Furthermore, it has been demonstrated that cortisol can be produced by the hair follicle in a functional equivalent to HPA axis (Ito *et al.,* 2005). For this reason, in order to avoid contamination from blood, and for the possible endocrine activity, it is important not to include the hair follicle in the sample. The main advantages of hair samples are that cortisol values are not affected by momentary stress and that it is a non-invasive and painless method. Furthermore, hair can be stored at room temperature before the analysis and small amounts of sample are sufficient (Gow *et al.*, 2010; Russell *et al.*, 2012; Van Uum *et al.*, 2008). One of the most remarkable inconveniences of hair cortisol analysis is that sampling requires validation for each species because of the interspecies differences in cortisol rhythm, secretion and stress response (Buchanan and Goldsmith, 2004; Keckeis *et al.*, 2012). Moreover, other confounding influences such as hair growth rate, gender, age, hair colour, environmental exposure and others may affect the results (Gow *et al.*, 2010). Nevertheless, according to Stalder *et al.* (2012), there is a high intraindividual stability in hair cortisol

concentrations. Different studies have used different hair cortisol analyses techniques: enzyme linked immunosorbent assay (ELISA) (e.g. Manenschijn *et al.*, 2011), Enzyme immunoassays (EIA) (e.g. Davenport *et al.*, 2006), radio-immunoassays (RIA) (e.g. Comin *et al.*, 2011) and high performance liquid chromatography-mass-spectrometry (HPLC-MS) (e.g. Gao *et al.*, 2010), being ELISA and EIA the most common. Furthermore, the association between cortisol concentration in hair and other biological samples has been investigated, finding correlation in faeces (Accorsi *et al.,* 2008), saliva (Davenport *et al.,* 2006; Bennett and Hayssen 2010) and urine (Sauvé *et al.,* 2007).

The main objective of this study was to analyse the feasibility and reliability of using hair as a matrix to determine the cortisol levels in growing pigs and if there were differences according to the region sampled.

2. MATERIALS AND METHODS

2.1. Animals and housing conditions

The study was performed with 56 crossbred [(LandracexDuroc) x Pietrain] entire males. The pigs were randomly selected from a group of 84 pigs belonging to a broader study aiming to determine the optimal number of pigs per pen for a precision feeder (Feeder type 1), which was contrasted with an already commercial electronic feeder system (Feeder type 2). No more than two piglets per litter were selected in order to ensure maximum genetic diversity.

Piglets were weaned at 21 days and were moved to the experimental farm at the age of six weeks and a mean weight of 13.78 kg. At the age of nine weeks and a mean live weight of 22.99 kg, piglets were randomly distributed in four fattening pens equal in size and form. During the first week, an additional hopper was installed inside each pen for an easier adaptation. The facilities where the experiment was carried out had automatic control system for temperature and ventilation, total slatted floor, and bowl type drinkers. Pigs were divided in two modules. Each module had two pens divided by a corridor which allowed visual contact between pigs in adjacent pens, and each pen had a computerized feeder. The size of the pen could be modified with two mobile fences, in half a pen, ¾ of pen and the entire pen. It allowed testing three different group sizes: 14, 21 and 28 pigs (G14, G21 and G28), respectively. The density was the same for each pen regardless of group size (0.99 m^2 /pig). Feeder was always in the pen, and there were one drinker in G14, two in G21 and three in G28 (Figure 1).

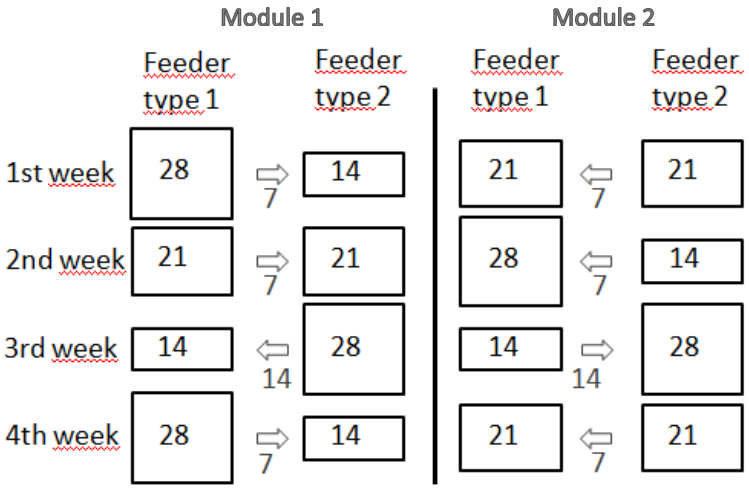

Figure 1: schematic representation of the animals' remixing schedule during the first four weeks. Module 1 and 2 consisted of two pens with variable size (represented by cubes) at each side of a central corridor). Animals were moved weekly between pens, and pens were resized in order to keep the stocking density. The bigger and smaller size of the cubes illustrates the size of the pen, and the numbers indicate the animals present in each pen. The arrows represent the movement of animals between pens from one week to the following one (e.g. Number 7 in week 1, means that 7 pigs were moved at the end of week 1 so that in week 2, 21 animals were in each pen of module 1).

Animals were weekly remixed in different groups and pen sizes were modified by moving the fences. Fourteen pigs were kept in the same pen during the whole experiment but the other 14 animals per module were moved each Friday from one pen to the other of the same module. Pigs which were not moved were considered the experimental pigs, and were sampled for hair cortisol analysis. Twelve rounds were needed in order to test these experimental pigs four times in each of the three group sizes (Figure 2). The 28 pigs moved (14 per module) from one pen to the other were not sampled for hair cortisol analysis, and they were only used to change the size of the groups.

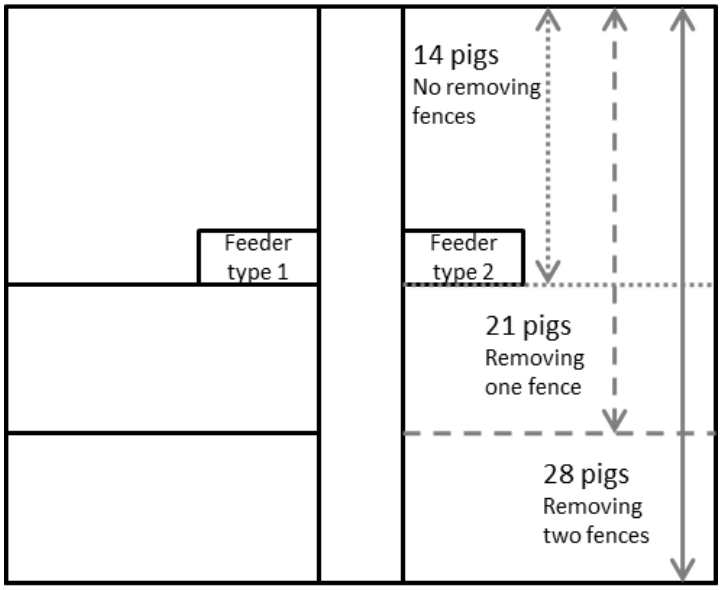

Figure 2: Schematic representation of pen size and location of mobile fences according to the number of pigs per pen. Fences were moved weekly to change the size of the pens according to the number of pigs moved.

2.2. Hair samples

Two hair samples were obtained from the same area, one in experimental week eight, when pigs were 17 weeks of age (sample 1) and one in week 12, when pigs were 21 weeks of age (sample 2). The samples were taken between 08:00 and 12:00. Animals were sampled taking advantage of the restrain provided by the cage of the scales in the regular weighing carried out every week. Pigs were gently accompanied to the scales, which had a two door system for access and exit. Samples were obtained from two locations, one in the dorsal area of the neck behind the ears (D) and the other in the loin part of the dorso–lumbar (L) region. Hair was collected by shaving close to the skin with clippers, trying not to remove the root of the hair, and avoiding including the hair follicle in the sample. Once sampled, hair was stored at room temperature inside hermetically sealed bags until analysis. Although 56 animals were assessed, only 49 were analysed. The criteria applied to discard an animal was the number of samples available (i.e. animals with not enough hair in a minimum of two samples were removed). Thirty-one valid samples for sample 1 region D, 42 for sample 1 region L, 35 for sample 2 region D and 47 for sample 2 region L were available. Furthermore, only 25 of the animals had enough hair in all regions both days.

2.3. Cortisol extraction from hair

Cortisol extraction was done following the method of Davenport *et al.* (2006) with a few modifications. First, approximately 150 mg of hair were washed twice in three mL of 99.5% isopropanol for 30 seconds to eliminate contaminants that can interfere with the determination (in case of smaller samples the volume of isopropanol was reduced in order to maintain the same proportion). Hair was then allowed to dry overnight in the airflow hood. The following day, samples were finely minced using surgical scissors until hair segments were 0.3 cm maximum length. For cortisol extraction, one mL of 99.5% methanol was added to approximately 50 mg of powdered hair, and it was incubated at 37 ºC during 17 hours with slow rotation. Then, the sample was spun in a microcentrifuge for 30 seconds at 5000 revolution per minute (rpm). At the end of extraction, the eppendorfs were centrifuged and 0.6 ml of the supernatants were finally dried using a vacuum centrifuge and stored at -20ºC. The dry extract was reconstituted in phosphate buffer solution from the assay kit.

2.4. Saliva samples

Saliva samples from 20 animals were collected the day before obtaining hair samples. All the samples were taken between 08:00 and 12:00 (starting at 08:00 all the days) to reduce the circadian rhythm effect. Saliva was collected with cotton buds Salivette® (SARSTEDT AG & Co, Nürbrecht, Germany) allowing the pig to chew it for one minute approximately. After the extraction, Salivettes® were centrifuged for ten minutes at 3500 (rpm), cotton buds were removed and tubes were frozen at -18 ºC until analyses were performed.

2.5. Cortisol assay

Both hair and salivary cortisol concentration were assessed using a High Sensitivity Salivary Cortisol enzyme immunoassay (EIA) kit (Salimetrics, State College, PA, USA) following the instructions provided by the manufacturer. For hair analysis, resulting values were converted from µg/dl to pg/mg of hair for data analysis. According to the manufacturer, cross-reactivity of the antibody with other steroids is as follows: prednisolone= 0.57%, cortisone= 0.13%, 11-deoxycortisol= 0.15%, 21-deoxycortisol= 0.04%, dexamethasone= 19.2%, triamcinolone= 0.09%, corticosterone= 0.21%, progesterone= 0.02%, and testosterone= 0.01%. There is no detectable cross-reactivity with prednisone, 17- hydroxyprogesterone, aldosterone, 17-β-estradiol and DHEA. Linearity was

evaluated with a sample of hair extract which was serially diluted. Recovery was determined by spiking a hair sample with a known amount of commercial cortisol before extraction (3 µg/dL). The lower limit of sensitivity (limit of detection) was determined by interpolating the mean optical density minus 2 SDs of 12 replicates at the 0 µg/dL level. The limit of quantification corresponds to the lower concentration of the cortisol in the calibration curve and represents the lower amount of cortisol which can be measured with total reliability. Assay precision was assessed by calculating intra-assay and inter-assay coefficient of variation in extracts at two ranges of cortisol concentration (low and high). Ten replicate samples from each concentration were analysed in the same ELISA plate for the intra-assay and two for the inter-assay for three days, and the coefficients of variation were calculated.

2.6. Statistical analysis

All the statistical analyses were conducted using Statistical Analysis System (SAS version 9.2; SAS Institute Inc., Cary, NC, USA). The significant level was established at $P<0.05$ for all the analyses. Unless otherwise indicated, results are presented as mean ± standard error mean (SEM) The variable cortisol was transformed into logarithms in order to obtain a Gaussian distribution.

A repeated measure analysis (PROC MIXED) was also done. The experimental unit was the animal, and the fixed effect was sample day nested by region. Box was found to have no significant fix effect in the model, so it was included in the model as a random effect. The best adjusted matrix correlation was obtained using the covariance structure Cs. The least square means of fixed effects (LSMEANS) were used when analysis of variance indicated differences. Correlations were analysed using a Parametric Pearson's rank correlation test between different days and regions, and between saliva and hair analysis.

3. RESULTS

3.1. Technical validation of the assay

The assay presented a very good linearity with a R= 0.9988 (Figure 3) and a recovery yield of 79.6% ± 3.2 assessed by spiking the sample before extraction with pure cortisol. The measured hormone concentrations in the spiked samples correlated with the expected concentrations (R= 0.9995) (Figure 4). The limit of detection was 0.017 µg/dL and the limit of quantification was 0.051 µg/dL.

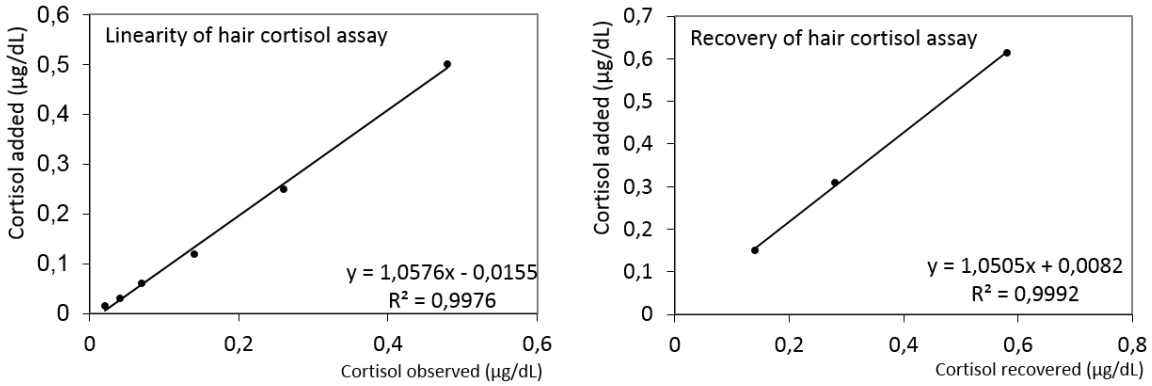

Figure 3 and 4: Linearity of hair cortisol assay. A sample with known concentration of cortisol was diluted 1:2, 1:4, 1: 8, 1:16, 1:32 and determined by ELISA. Recovery of exogenous cortisol from pig hair samples compared to the amount of commercial cortisol added before extraction.

Assay precision was assessed by calculating intra-assay and inter-assay coefficients of variation in hair extracts at two ranges of concentration. Intra-assay variation (variation within plates) ranged from 8.45% (low concentration, 0.28 µg/dL) to 3.75% (high concentration, 0.99 µg/dL). Inter-assay variation (variation between plates) ranged from 10.9% (low concentration, 0.084 µg/dL) to 6.7% (high concentration 0.26 µg/dL).

3.2. Biological validation of hair cortisol as stress marker

Hair cortisol values were extremely variable across different animals, with a minimum of 6.4 pg/mg of hair and a maximum of 43.88 pg/mg and a coefficient of variation of 41%. The average value for all the samples was 19.30 ± 0.63 pg/mg. The mean value for sample 1 (both D and L) was 17.69±1.17 pg/mg (n=73), and for sample 2 was 20.35 ± 1.14 pg/mg (n=82). When comparing among the regions, the mean for region D (both sample 1 and sample 2) was 17.05 ± 1.20 pg/mg (n=66) and for region L the mean was 20.97 ± 1.123 pg/mg (n=89). Results of the different regions and different days

sampled and logarithmic transformation of data and P-values for different comparisons are presented in Table 1. Repeated measure analysis for hair samples showed significant differences between different sampled days, showing a mean increase over the time. Differences between the regions sampled were also found, being the hair samples obtained from the L region 3.93 pg/mg higher in average (P= <0.001) than those obtained for the D region. In this case, significant differences were found for both sample 1 and sample 2, being higher in the region L for both days (the average difference was 4.79 pg/mg for sample 1 and 3.1 pg/mg for sample 2).

Table 1: Descriptive statistics, logarithmic transformation and p-values for different regions and different days of cortisol levels in pig's hair (pg/mg).

	D region				L region				P-value
	X	SEM	min-max	n	X	SEM	min-max	n	Region[2]
Sample 1	15.31	1.42	6.86-33.96	31	20.06	1.29	9.00-43.88	42	0.0011
S1 log	2.65	0.07	1.93-3.53	31	2.92	0.06	2.20-3.78	42	
Sample 2	18.80	1.37	6.40-40.00	35	21.90	1.25	7.00-37.50	47	0.0269
S2 log	2.85	0.07	1.86-3.69	35	3.03	0.06	1.95-3.62	47	
P-value Sample[1]			0.0441				0.2762		

X=mean, SEM= Standard error of the mean, min-max=minimum and maximum (all expressed in pg/mg), n=number of adequate samples, sample 1= week 8 of experiment, S1 log= logarithmic transformation of sample 1, sample 2= week 12 of experiment, S2 log= logarithmic transformation of sample 2, D region= hair from dorsal area of the neck, L region= hair from lumbar region.

[1] P-values between sample 1 and 2 within each region.

[2] P-values between region D and L within each sample.

3.3. Salivary cortisol

Salivary values ranged from 0.60 to 6.90 ng/mL, and the coefficient of variation was 54%. No significant differences were reported among different samples. The average value for all the samples was 1.81 ng/mL with a mean Standard Error of 0.13 ng/mL. The first and second sample means were 1.83 ± 0.13 ng/mL and 2 1.92 ± 0.29 ng/mL, respectively.

3.4. Correlations

No correlations between salivary samples and hair samples were reported. Correlations for hair samples are shown in Figure 5. Significant positive correlations were found between first sampled day and second sampled day in both region D. There were also correlations between both regions for sample 1 and sample 2. A correlation was also found between L region sample 1 and D region sample 2.

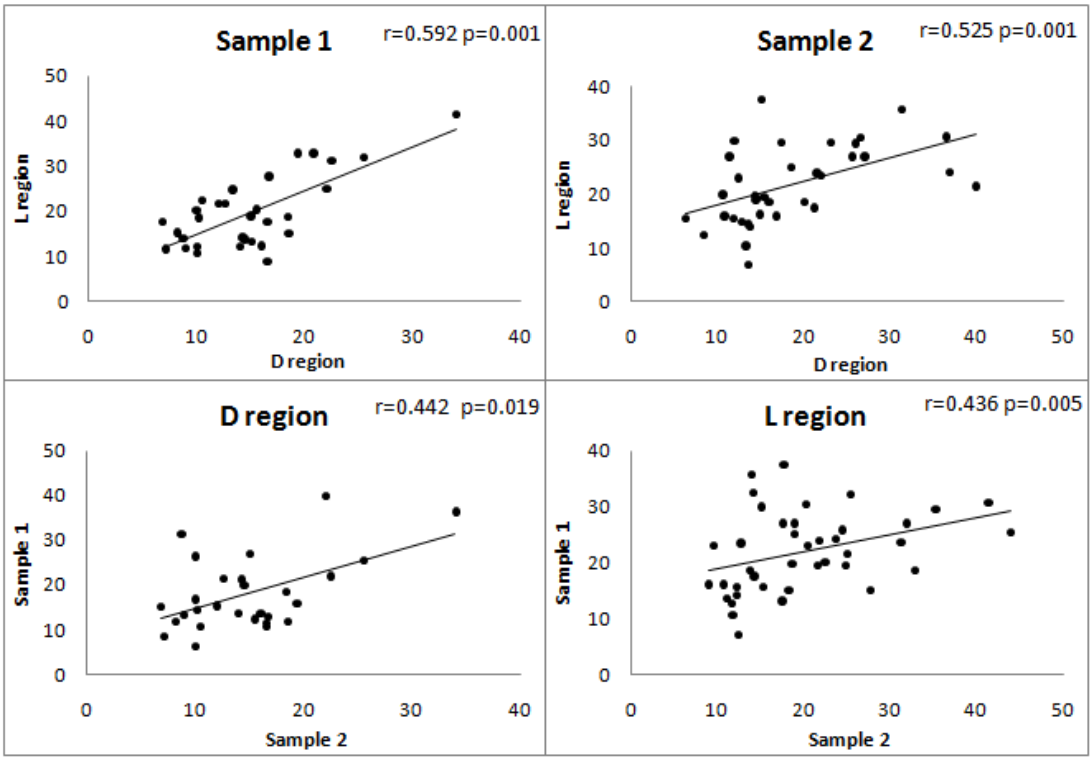

Figure 5: Correlations of pig's hair samples between different regions (L region for dorso-lumbar region and D region for dorso-lumbar region) and sampled days (Sample 1 for week 8 of experiment and Sample 2 for week 12 of experiment). r= correlation coefficient, p= P-value.

4. DISCUSSION

In this study we analysed the feasibility and reliability of using hair as a matrix to determine the cortisol levels in growing pigs and the differences between the regions sampled.

4.1. Validation of the assay

Linearity, recovery and intra-assay and inter-assay coefficients of variation were similar to those described for other species using similar extraction procedures and ELISA-based cortisol assay (Davenport *et al.,* 2006; Bennett and Hayssen 2010; Manenschijn *et al.,* 2011; Yamanashi *et al.,* 2013).

4.2. Cortisol levels in hair samples

Cortisol levels obtained in this experiment were, in general, slightly higher than those previously reported in pigs. Along that line, Martelli *et al.* (2014) reported lower levels in transgenic pigs and their relatives, while Bacci *et al.* (2014) found a clear influence of the reproductive phase in sows, with similar levels at the diagnosis of pregnancy, but lower levels before delivery. A high variability in cortisol levels was also found as reported in other studies (Suavé *et al.*, 2007). Salivary and blood cortisol levels have traditionally been reported as presenting a high individual variation (Mendl, 1991; Rushen, 1991; Fàbrega *et al.*, 2002). It was expected that the variance when evaluating cortisol in hair was lower compared to salivary or blood cortisol, and, thus, the present findings could be partially attributed to some methodological aspects further discussed below. However, more studies would be recommended to better understand the individual variability of hair cortisol. Furthermore, the levels of cortisol were found significantly higher in sample 2. One possible hypothesis to explain this increase throughout the time is related with the continue restructuration of the groups. As it has been reported in previous studies, regrouping and mixing growing pigs may induce chronic stress (e.g. Coutellier *et al.*, 2007). Furthermore, although this hypothesis should be confirmed with further studies, basal cortisol levels tend to decrease with age (de Jong *et al.*, 2000; Ruis *et al.*, 1997), supporting that the increment observed in hair samples could be triggered by the remixing procedure and disentangled from the effect of ageing. However, it is also true that caution is necessary to compare cortisol levels in hair over time, since it is meant to reflect accumulation and, thus, factors like age of hair may influence and be difficult to disentangle from stress itself.

Cortisol was found to be significantly higher in L than D region. These differences between regions had been described in other species such as grizzly bears (*Ursus arctos*) (Macbeth *et al.*, 2010), Alaskan Caribou (*Rangifer tarandus granti*) ((Ashley *et al.*, 2011), Canada lynx (*Lynx canadensis*) (Terwissen *et al.*, 2013), chimpanzees (*Pan troglodytes*) and also in Humans (Li *et al.*, 2012) suggesting that the effect of the region sampled is important and should be taken into account. Several hypotheses could explain this finding. One possible explanation for the intraindividual differences within the same day could be associated with rhythm of hair growth and shedding, being faster for D compared to L region. This could be because either growth may have started before in some regions than in other ones, resulting, in turn, in earlier accumulation of cortisol in those regions, or even if growth started simultaneously, subsequent growth rhythm differed between regions. No studies on rhythm of hair growth in pigs have been found to compare these results. Another hypothesis could be the pigmentation of the hair. Bennett and Hayssen (2010) and Yamanashi *et al.* (2013) have reported different cortisol levels depending on the coat colours, finding more cortisol in light colours than in dark. Some of the pigs sampled in this experiment did not have uniform coat colour, what could be a potential explanation of the differences found. Finally, another explanation could be related with the multi-comportment model suggested by Henderson (1993). According to that model, substances can be incorporated to the hair by external sources, and considering the possibility that lumbar region may present more dirt than dorsal area of the neck, more cortisol from the faeces might be incorporated in this particular area. Hair cortisol levels increased also significantly in D region throughout time (P=0.044), whereas for L region there was a tendency to increase over time, but this increase was not significant (P=0.185). More studies should be carried to try to explain this finding, in order to better clarify the importance of region on sampling and understand whether the accumulation mechanisms could differ.

The authors would like to point out some methodological aspects, like the amount of hair available, although they have not been reported as a problem in other species. In some breeds of pigs it may be difficult to obtain the intended amount of hair, especially in young piglets. Most piglets at birth and in their first weeks of age have small amounts of hair, not enough to obtain sufficient sample. In this study, differences of amount of hair between the first sample and the second one were found, being more difficult to achieve the needed sample size in the younger animals. Furthermore, after the sample collection, some animals had to be discarded because of the lack of sample, especially in the region D (in L region was easier to obtain the necessary amount of hair needed) Due to these difficulties, gentle restraining of pigs during the sample procedure, especially those with small amounts of hair may be required.

4.3. Correlations between samples

No correlations were found between hair cortisol levels and saliva samples. The hypothesis to explain this lack of correlation may be that cortisol in saliva can be detected after a few minutes, but it accumulates over days in hair. Our results are in accordance with those obtained for Sauvé *et al.* (2007). Furthermore, this hypothesis is supported by the study of D'Anna-Hernandez *et al.* (2011) in which correlations were found only when samples taken over a long period of time were considered. More saliva samples during a longer period are strongly recommended for studying the correlation between saliva and hair. Both correlations between regions and time were significant but not as strong as expected. On the contrary, Stalder *et al.* (2012) found good intraindividual correlation between repeated hair analyses in humans' hair (between r=0.68 and r=0.79), better correlations were also found in chimpanzees' hair (between r=0.61 and r=0.71) by Yamanashi *et al.* (2013). Our main hypothesis to explain why in our study the correlations were lower may be related to the sampling procedure. A possible explanation is that the sampled region was not shaved before sampling and, thus, it is not possible to ascertain whether the second sample was taken exactly from the same hair as the first one. This may have an effect, since if cortisol in hair is believed to accumulate over time, cutting exactly the same hair may have an effect on the accumulated level. This hypothesis is supported by the fact that the correlations between the regions of the second sampling day are lower than between the first day. For this reason, cutting hair from the region to sample previous to the first sample (with enough time to let the hair grow, approximately 1 month) and before every sample (to ensure that new hair is sampled each time) may be strongly recommended. Another alternative could be to obtain samples from a very proximal region, trying not to collect hair of the same region. Other hypothesis to explain the low correlations between regions in both days could be the potential differences in growth rhythm previously mentioned, because of the intraindividual difference in the coat color for the different regions, or because intraindividual hair cortisol stability in pigs is not as strong as in other species.

5. CONCLUSIONS

The results obtained in the present study confirmed, as found in previous studies, that it is possible to detect cortisol in growing pigs' hair. However, further studies with a higher number of individuals would be required to better understand its mechanisms before recommending its use as a chronic stress indicator. Significant differences depending on the region sampled were found, suggesting that this should be taken into account. It seems recommendable to sample the same region, cutting

the hair at the beginning and after each sampling, to ensure that the hair growth will be the same, and hence, the period of time analysed will be compressed between the shaving and the sample collection. In pigs, especially in very young animals, it could be difficult to obtain the amount of hair needed, and it is recommended to gently restrain the animals.

6. ACKNOWLEDGEMENTS

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Chapter 2

Salivary chromogranin A and hair cortisol as chronic stress indicators in growing pigs

Based on a paper submitted to Animal, under review

ABSTRACT

Stress response induces physiological, behavioural, immunological and biochemical changes that directly affect health and well-being. Although these changes can be assessed using different biomarkers, there is a lack of consistent data regarding markers for chronic stress assessment. The aim of this study is to analyse the use of cortisol concentration in hair, chromogranin A (CgA) in saliva and Tumour necrosis factor-alpha (TNF-α) in blood as chronic stress biomarkers, after using two strategies for minimizing stress: enrichment material (EE) and/or an herbal compound (HC). For this purpose, 56 pigs were divided into eight groups of seven pigs (14 pigs/treatment): a) two groups provided with EE, b) two groups supplemented with HC, c) two groups provided both with EE and HC, d) and two control groups (CG). Samples of hair, saliva and blood were taken to measure cortisol, CgA and TNF-α at three different times: before starting the experiment (T0), and after one (T1) or two months (T2). No differences were found at T0 in cortisol, CgA or TNF-α, whilst at T2, the control group showed significant increased concentrations, when compared with the rest of the treatments ($p<0.001$) in both cortisol and CgA but not in TNF-α. These differences were significant at T1 only for CgA ($p<0.001$). Furthermore, a correlation was observed between hair cortisol and salivary CgA ($r=0.48$, $p<0.001$). These results support hair cortisol and suggest chromogranin A as useful tools to detect chronic stress in growing pigs.

1. INTRODUCTION

The interest in techniques (especially non-invasive) to objectively assess animal welfare and particularly stress response, has increased during the last few decades. Stress response produces biochemical changes by the activation of the sympathetic adrenomedullary (SAM) system in a first phase and the hypothalamic-pituitary-adrenal (HPA) axis after a short delay (Manteuffel, 2002). As a consequence of the activation of the SAM system, chromogranin A (CgA) is co-released with catecholamines (adrenaline and noradrenaline) (O'Connor and Frigon, 1984), while the main output of the HPA axis in pigs is cortisol (Mormède *et al.*, 2007). Thus, CgA and cortisol have been used in pigs as physiological indicators of the SAM system and HPA axis activity, respectively (e.g. Escribano *et al.,* 2012 and 2013). On the other hand, stress-induced immunosuppression has also been widely reported, affecting the release of pro-inflammatory cytokines such as TNF-α and suggesting neuroendocrine mechanisms somehow meditated by glucocorticoids and catecholamines (Connor *et al.*, 2005).

Cortisol is the major glucocorticoid in most mammals including pigs. The increase of cortisol levels in biological samples such as plasma, saliva, urine, faeces and milk between a few minutes and several hours post stress (Mormède *et al.*, 2007) has been associated with acute or sub-acute stress. On the contrary, hair cortisol has been suggested as a long-term indicator of stress exposure (Davenport *et al.*, 2006; Gow *et al.*, 2010). Although there are still some gaps to completely understand the mechanism for cortisol incorporation into hair, the most accepted theory is the model suggested by Henderson *et al.* (1993). In this model, substances are suggested as being incorporated into hair via the blood during the formation of the hair, through sweat and sebum secretions, or deposited from the external environment. If hair cortisol is confirmed as a proper chronic stress indicator, it has important advantages, since hair sampling is a non-invasive and painless method and can be carried out by a non-professional (Gow *et al.*, 2010)

CgA is an acidic soluble protein which is the major member of the granin family (Hendry *et al.*, 1995). CgA is stored and co-released with catecholamines to the blood from the vesicles of the adrenal medulla, the sympathetic nerve endings and neuroendocrine tissues (O'Connor and Frigon, 1984; Obayashi, 2013). Furthermore, Saruta *et al.* (2005) described the production of CgA from the submandibular glands of humans, and different studies have found a relation between physical or psychological stress and CgA levels in saliva (*e.g.* Kanamaru *et al.*, 2006; Ott *et al.*, 2014; Escribano *et al.*, 2015). Salivary CgA seems to peak after a few minutes, but it lasts for up to one hour in humans

(Obayashi, 2013) and does not present a circadian rhythm in pigs (Escribano *et al.*, 2014). However, CgA has not been, up to now, investigated as a possible chronic stress indicator.

Growing pigs in modern pig husbandries are exposed to many stress factors which have a negative effect on their health and welfare. Thus, one of the key points in husbandry is to assess and reduce the stress level of pigs. Different strategies can be used when trying to minimize stress, and this study has focused on two of them. On one hand, some studies have demonstrated that barren conditions, compared to enriched environments, are associated with signs of chronic stress (Munsterhjelm *et al.*, 2010). Therefore, the provision of different environmental enrichment materials to allow the fulfilment of exploratory behaviour needs has been used as the first strategy of this study for stress reduction. On the other hand, the second strategy was based on the use of plants with sedative or relaxing properties. *Valeriana officinalis* and *Passiflora incarnata* have been reported to have such sedative and anxiolytic properties (Soulimani *et al.*, 1997; Peeters *et al.*, 2004; Nam *et al.*, 2013). Both plants are the main active components of the commercial herbal product used in this experiment (Sedafit).

The aim of this study is to analyse the use of cortisol concentration in hair and CgA in saliva as possible chronic stress biomarkers. For this purpose, the evolution of these analytes in relation to two different strategies of stress reduction, provision of enrichment material and/or supplementation of an herbal compound, was evaluated. Hair cortisol and CgA levels were contrasted with cortisol in saliva and TNF-α in blood, which is considered a biomarker of the immune function.

2. MATERIALS AND METHODS

2.1. Animals and housing conditions

Fifty-six crossbred [(Landrace x Large white) x Pietrain] entire males from the same genetic company were used in this experiment. No more than two piglets per litter were selected in order to ensure maximum genetic diversity. Weaners arrived at the experimental farm in December at the age of 10 weeks and an average weight of 25 kg. The facilities where the experiment was carried out had an automatic control system for temperature and ventilation, total slatted floor, and bowl-type drinkers. At the age of 16 weeks, and a mean live weight of 49.8 kg, pigs were randomly distributed into a total eight groups of seven animals, located in two modules. Each module had four pens, two on the left and two on the right separated by a corridor. Pens were divided using metallic-bar fences to allow direct visual contact. Each pen was 13.67 m^2 (5mx2.8mx4.3mx2m), allowing a floor space

availability of 1.95 m^2/pig. All pens were provided with one drinker and two hoppers, and water and food were supplied *ad libitum*.

Pigs were reared in either an enriched environment (EE) consisting of sawdust, natural hemp ropes and rubber balls (all materials were provided at the same time) or in a barren environment (BE). Furthermore, half of the groups were also supplemented with an herbal compound (HC+) containing Valerian (*Valeriana officinalis*) and Maypop (*Passiflora incarnata*), both having sedative effects (Sedafit; Phytosynthèse, Saint-Bonnet de Rochefort, France), while the others were not supplemented (HC-). Thus, four different treatments were assessed in both modules: EE and HC+, EE and HC-, BE and HC+, and BE and HC- (control group) (Figure 1). The slats of the pens supplied with EE were partially covered (1/3) with polypropylene sticks (Click-in®; Rotenca, Agramunt, Spain) to support the sawdust. New sawdust was added every two days. The hemp ropes were attached on the walls of EE pens, and were periodically substituted when the length of the rope after its use by the pigs was less than 30 cm. A single rubber ball with a diameter of 15 cm was provided in each EE pen. Sedafit was manually added to the HC+ groups in a concentration of 2000 mg/Kg to the food concentrate, while HC- pigs were supplied with normal food concentrate without supplementation. Blood, saliva and hair samples were taken before starting the experiment (T0) (16 weeks of age), at the middle (T1) (20 weeks of age) and at the end (T2) (24 weeks of age). Furthermore, at the ages of 15 weeks, 18 weeks, 20 weeks, 22 weeks and 24 weeks, all animals were weighed and body lesions were assessed as part of a broader investigation, which also included weekly behavioural observations. All procedures carried out during this experiment were approved by the IRTA ethical committee.

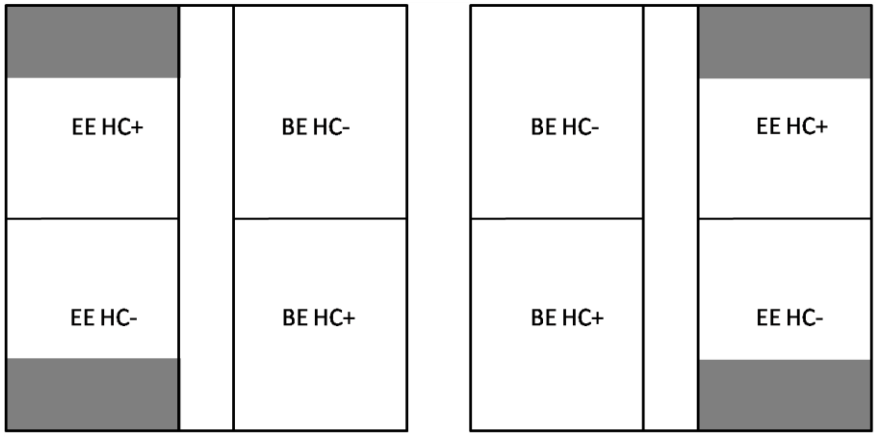

Figure 1: Schematic representation of the experimental pig farm and distribution of the two rooms used: EE= provided with enrichment material BE= Barren environment; HC+= provided with herbal compound (Sedafit); HC-= not provided with herbal compound. BEHC-= control group. The grey area represents the slats covered to support sawdust.

2.2. Hair collection and analysis

Hair was sampled taking advantage of the restrain provided by a scale with a cage and a two-door system for access and exit used for the regular weighing carried out every two weeks. Clean hair was carefully shaved using a hair trimmer, trying not to include the hair follicles, which have been described as producing cortisol themselves (Ito *et al.,* 2005). The samples were obtained from the lumbar region, which was suggested in a previous study as adequate (Casal *et al.*, 2014). The lumbar region plus a security margin was shaved when the animals arrived at the experimental farm and after every sample procedure in order to ensure that the period of time analysed was comprised between the shaving and the sampled day. Pigs were gently accompanied to the scales, which had a two-door system for entrance and exit, and the hair trimmer was cleaned after each animal. Once sampled, hair was stored at room temperature inside hermetically sealed bags until analysis. Although 56 pigs were sampled, five samples in T0, and three in T1 and T2 were discarded because of the small amount of sample.

Cortisol extraction was performed following a method developed by Tallo-Parra *et al.* (2015), based on the method of Davenport *et al.* (2006). Approximately 250 mg of hair from each sample were washed in 2.5 mL of 2-propanol, vortexed at room temperature for 2.5 min, and the supernatant was discarded by decantation. The same washing process was repeated twice more. Hair samples were then allowed to dry completely at room temperature for approximately 36 hours; then,

samples were powdered using a ball mill (Mixer mill MM200; Retsch, Haan, Germany) for five minutes at 25 Hz. Fifty mg of powdered hair per sample were placed in 2-mL eppendorf tubes with 1.5 mL of methanol and moderately shaken at 30ºC for 18 hours (G24 Environmental Incubator Shaker; New Brunswick Scientific Co. Inc., Edison, NJ, USA) to extract the steroids. Then, the sample was spun in a micro centrifuge for 2 min at 7000 g, and 0.750 mL of each sample were aliquoted into a new 2-mL eppendorf and dried at 38ºC in an oven. The dried extracts were reconstituted with 0.2 mL of the solution provided with the cortisol assay kit, and stored at −20°C until the analysis.

Hair cortisol concentration was analysed using an enzyme-linked immunosorbent assay (ELISA) kit *(*Cortisol ELISA KIT; *Neogen®* Corporation Europe, Ayr, UK) following the instructions provided by the manufacturer. All samples were analysed in duplicate. Validation was necessary since this kit is not specific for pigs and it had not been tested before. The sensitivity of the kit was of 0.32 pg cortisol/mg of hair. Validation tests were performed using pools of 10 different samples. According to the manufacturer, cross-reactivity of the ELISA antibody with other steroids was as follows: prednisolone 47.4%, cortisone 15.7%, 11-deoxycortisol 15.0%, prednisone 7.83%, corticosterone 4.81%, 6β-hydroxycortisol 1.37%, 17-hydroxyprogesterone 1.36%, deoxycorticosterone and <1% for the rest of the steroids not presented. Specificity was also evaluated comparing slopes from the straight lines resulting from the standard curve ($m_{standard}$) and a new pool curve (m_{pool}) with the same serial dilutions (1:1, 1:5, 1:10, 1:25, 1:50 and 1:100). Linearity was evaluated with pooled samples serially diluted (1:1, 1:2, 1:5 and 1:10) in the buffer kit, showing a R^2= 0.972 and a mean percentage error of -18.9%. Accuracy was assessed through the spike-and-recovery test: 50, 100, and 200 µl of pure standard cortisol solutions were added to 200, 100 and 50 µl of pool sample, respectively; this process was repeated at different standard cortisol concentrations (20 ng/mL, 2 ng/mL and 0.2 ng/mL), and the percentage of recovery was 113.67% ± 14.15 % SEM. Assay precision was assessed by calculating the intra-assay and inter-assay coefficient of variation. The intra-assay coefficient of variation was calculated from all duplicated samples, with a result of 2.33%, while for the inter-assay it was calculated from a pool of 10 high-variability samples duplicated in each ELISA plate, with a result of 11.83%.

2.3. Saliva collection

Saliva samples were taken allowing the animals to chew on one cotton bud (Salivette®; SARSTEDT AG & Co., Nürbrecht, Germany) for more than 30 seconds. Cotton buds were offered using a clamp. Both cortisol and CgA were assessed from the same samples. If the amount of saliva collected was

not sufficient for both analyses, CgA was considered the priority. At the age of 16 weeks, saliva was collected from outside the pen. When it was not possible, samples were obtained from inside the pen, avoiding restraining the animals. Sampling time was fixed up to five minutes. Although all animals were correctly sampled, in 10 pigs (4 EEHC+, 1 EEHC, 3 BEHC+, and 2 BEHC-) the amount of saliva was not sufficient for both cortisol and CgA, and hence, cortisol was not assessed. For the 2nd and 3rd samples, saliva was collected at the same moment as was blood by two different operators, taking advantage of the pigs being restrained. All animals had enough sample for CgA analysis, but at the age of 20 weeks, two pigs did not have enough sample for cortisol determination (1 BEHC+ and 1 BEHC-). Once sampled, and before the analysis, salivettes® were centrifuged at 3500 rpm for 10 minutes, cotton buds were removed, and tubes were frozen at -18ºC.

2.4. Salivary cortisol analysis

Salivary cortisol concentration was measured using a solid-phase competitive chemoluminescent enzyme immunoassay kit (Immulite 1000 cortisol; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) validated for porcine saliva (Escribano *et al.*, 2012).

2.5. Salivary chromogranin A analysis

Salivary CgA was measured using a validated time-resolved immunofluorometric assay (TR-IFMA) (Escribano *et al.*, 2013) that performs having a lower limit of detection of 4.27 ng/mL and a high level of precision (inter-assay coefficient of variation) of 6.23% and intra-assay coefficient of variation of 5.82%). The coefficient of correlation for accuracy was r=0.968. The calibration curve covered a range from 46.8 ng to 1500 ng of CgA/mL. Fluorescence was measured using a VICTOR2 1420 multi-label counter (Perkin Elmer Life and Analytical Science; WallacOy, Turku, Finland).

2.6. Blood collection and analysis

All blood samples were taken via jugular vein puncture between 08:00 and 12:00 a.m. Once sampled, blood was centrifuged for 10 minutes at 3500 rpm, aliquoted and stored at -18ºC until analyses were performed. TNF-α levels were measured using a validated porcine TNF-α *ELISA kit* (Quantikine®; R&D Systems, Minneapolis, MN, USA).

2.7. Statistical analysis

All statistical analyses were conducted using the Statistical Analysis System (SAS version 9.2; SAS Institute Inc., Cary, NC, USA). The significant level was established at $p<0.05$ for all the analyses, and a tendency was considered between $p>0.05$ and $p<0.1$. Results are presented as mean ± standard error mean (SEM) unless otherwise indicated. The Shapiro-wilk test (with Proc univariate in SAS) was used to examine the normality of distributions. For data without a Gaussian distribution, a logarithmic transformation was performed, and all was evaluated again. One outlier was eliminated from the second sampled day for CgA.

Data were analysed using generalized linear mixed models for repeated measurements (glimmix procedure). The experimental unit was the subject. Use or not of enrichment material, and herbal compost, sample day and their interactions were considered as fixed effects. Subject nested with pen was considered as a random effect, after testing that the pen was not significant as a fixed effect. Sample day provided the repeated measurement. Least square means of fixed effects (LSMEANS) (i.e. estimated marginal means or means adjusted by a covariate) with Tukey adjustment were used for comparisons when analysis of variance indicated significant differences. Parametric Pearson´s rank correlations were calculated between different physiological indicators and days using the Proc Corr procedure of SAS.

3. RESULTS

3.1. Hair cortisol

Hair cortisol was significantly lower for pigs raised in EE and for pigs supplemented with HC+ at T2 (Tables 1 and 2, respectively). Hair cortisol concentrations and differences between treatments for each sampled day are presented in Figure 2. No significant differences between treatments were observed for T0 and T1, while the control group was significantly lower in relation to the other treatments at T2 ($p=0.004$ for BEHC+ and $p<0.001$ for EEHC- and EEHC+). Comparing the initial and final levels of hair cortisol, a significant decrease was observed between T0 and T2 for all three treatments ($p<0.001$ for BEHC+, EEHC- and EEHC+). These differences were significant between T0 and T1 for all groups, including the control group ($p=0.01$ for control group and $p<0.001$ for the other treatments), but between T1 and T2, the only group with a significant decrease were those provided with both enrichment and herbal compound ($p=0.006$).

Table 1 and table 2: Hair cortisol, CgA, salivary cortisol and TNF-α concentrations of the different sampled days for the growing pigs kept in barren conditions or environmentally enriched conditions (table 1), and supplemented or not with an herbal compound (table 2).

	BE			EE				P-values		
	T0	T1	T2	T0	T1	T2	SEM	eet	Sday	eetxsday
Hair cortisol (pg/mg hair)	11.99^{a}	6.78^{b}	7.58^{b}	12.63^{a}	5.53^{b}	3.72^{c}	0.40	0.040	<0.001	<0.001
CgA (µg/mL saliva)	1.14^{a}	0.82^{ab}	0.54^{bd}	1.11^{a}	0.37^{cd}	0.26^{c}	0.05	0.0013	<0.001	0.0107
Salivary cortisol (µg/dL saliva)	0.61	0.6	0.41	0.63	0.38	0.43	0.03	n.s.	0.016	n.s.
TNF-α (pg/mL blood)	116.88	87.56	63.65	108.35	84.16	57.31	2.81	n.s.	<0.001	n.s.

	HC+			HC-				P-values		
	T0	T1	T2	T0	T1	T2	SEM	hct	sday	hctxsday
Hair cortisol (pg/mg hair)	11.33^{a}	6.47^{b}	7.27^{b}	12.26^{a}	5.80^{b}	4.19^{c}	0.40	0.32	<0.001	<0.001
CgA (µg/mL saliva)	1.06^{a}	0.84^{b}	0.52^{bd}	1.20^{a}	0.35^{cd}	0.28^{c}	0.05	0.0285	<0.001	0.0164
Salivary cortisol (µg/dL saliva)	0.56	0.50	0.43	0.70	0.48	0.41	0.03	n.s.	0.016	n.s.
TNF-α (pg/mL blood)	117.02	86.15	61.05	108.21	85.5	59.77	2.81	n.s.	<0.001	n.s.

eet= Environmental enrichment treatments. BE=Barren environment, EE= Enriched environment.
hct= Herbal compound treatment; HC+= supplemented with a commercial herbal compound with *Valeriana officinalis* and *Passiflora incarnata;* HC-= not supplemented.
sday= sampled day: T0=before experiment, week 16, T1= after 1 month of treatment, week 20, T2= after 2 months of treatment, week 24.
^{a,b,c,d} Values within a row with different superscripts differ significantly at $P<0.05$, according to the Lmeans of the interaction (eet x day). ns: P> 0.05.

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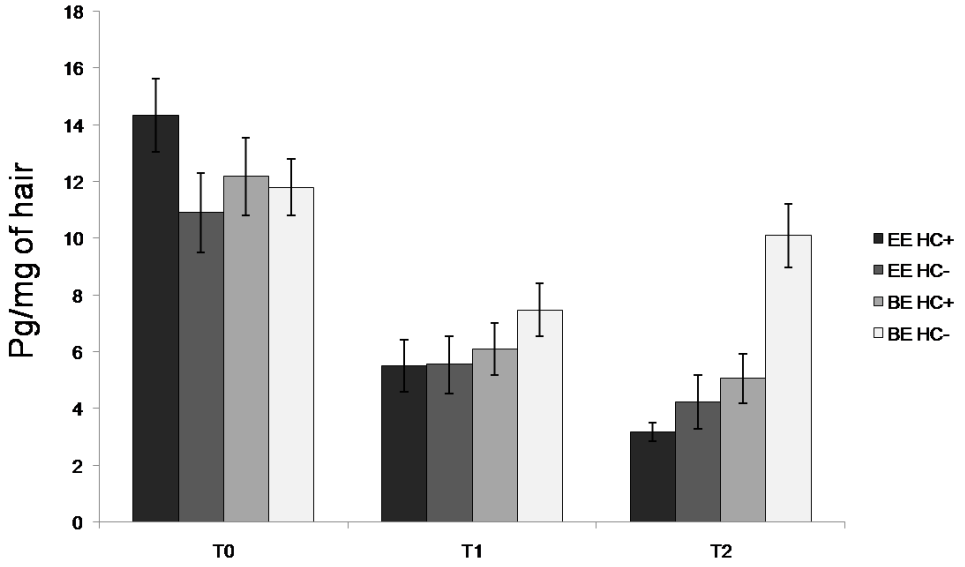

Figure 2: Hair cortisol concentrations (mean and SEM) of pigs grown in enriched environment conditions (EE) or barren conditions (BE) and supplemented (HC+) or not (HC-) with an herbal compound before starting the experiment (T0, 16 weeks of age), after one month of treatment (T1, 20 weeks of age) and after two months of treatment (T2, 24 weeks of age).

3.2. Chromogranin A

Salivary CgA was significantly lower for pigs with EE and for HC+ at both T1 and T2 (Tables 1 and 2, respectively). Salivary CgA concentrations for each treatment and day are expressed in Figure 3. No significant differences between treatments were observed for T0, while CgA concentrations were different at T1 and T2 for the control group, when compared to the rest of the treatments (at T1: $p<0.001$ for EEHC- and EEHC+, and $p=0.002$ for BEHC+; at T2: $p<0.001$ for EEHC- and BEHC+, and $p=0.02$ for EEHC+). When comparing over time, significant differences were observed between T0 and T2 and between T0 and T1 for all treatments ($p<0.001$) except for the control group. The only differences observed between T1 and T2 were a slight decrease for BEHC+ ($p=0.036$) and a tendency to decrease for EEHC- ($p=0.10$).

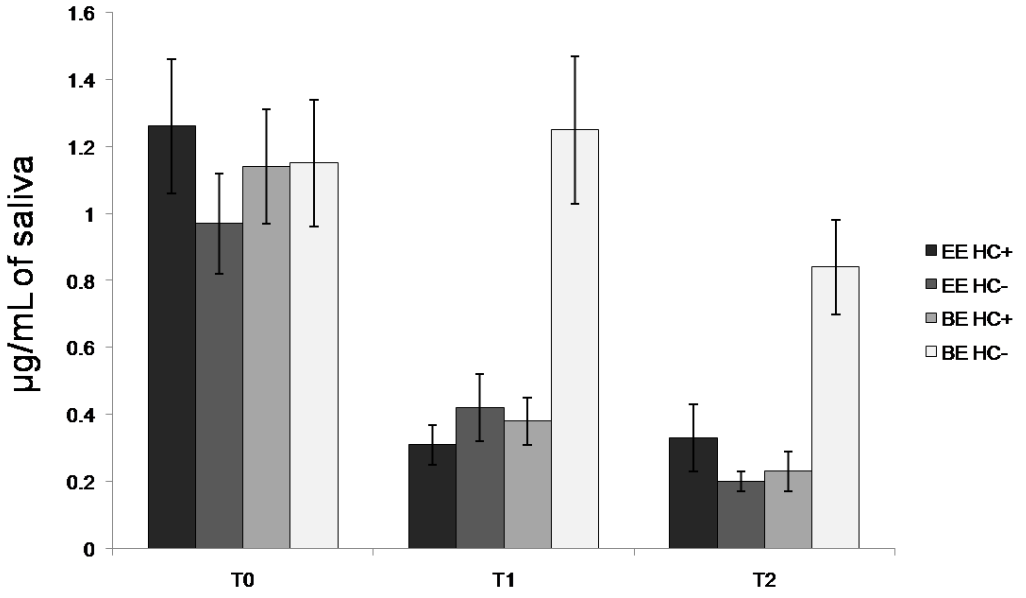

Figure 3: Salivary chromogranin A (CgA) concentrations (mean and SE) of pigs grown in enriched environment conditions (EE) or barren conditions (BE) and supplemented (HC+) or not (HC-) with herbal compound before starting the experiment (T0, 16 weeks of age), after one month of treatment (T1, 20 weeks of age) and after two months of treatment (T2, 24 weeks of age).

3.3. Salivary cortisol and serum TNF-α

Neither enrichment nor Sedafit resulted in significant differences for salivary cortisol and TNF-α concentration (Tables 1 and 2). However, a significant decrease was observed between T0 and T2 in both salivary cortisol ($p=0.02$) and TNF-α ($p<0.001$).

3.4. Correlations

Hair cortisol was positively correlated with CgA ($r=0.48$, $p<0.0001$) and TNF-α ($r=0.33$, $p<0.0001$), but not with salivary cortisol. CgA, apart from hair, was also correlated with salivary cortisol ($r=0.27$, $p=0.0007$) and TNF-α ($r=0.36$, $p<0.0001$). Contrasting the different sampled days, hair cortisol correlations were found between T0 and T1 ($r=0.41$, $p=0.0031$) and T1 and T2 ($r=0.57$, $P<0.0001$), and CgA presented a significant correlation between T1 and T2 ($r=0.42$, $p=0.0016$). At T0, CgA was correlated with salivary cortisol ($r=0.58$, $p<0.0001$). Moreover, hair cortisol and CgA presented a significant correlation at T2 ($r=0.40$, $p=0.0024$).

4. DISCUSSION

Non-invasive physiological indicators to evaluate stress have been widely investigated during the last few decades in different species. The present research has focused on changes in salivary CgA and hair cortisol as indicators of chronic stress, when using two strategies aimed at stress reduction. With this purpose, different groups were reared in different conditions including enrichment material and supplementation of an herbal compound (Sedafit), both suggested as potential reducers of stress. Along that line, environmental enrichment has been related with a reduction of the stress and an improvement of the welfare of growing pigs (Van de Weerd and Day, 2009; Young, 2003). Concerning the herbal compounds, the sedative and tranquilizing properties of *Valeriana officinalis* and *Passiflora incarnata* (the main components of Sedafit) have previously been described (Soulimani *et al.*, 1997; Nam *et al.*, 2013). Furthermore, Peeters *et al.* (2004) reported a reduction of the stress response in pigs supplied with this product. Therefore, in this experiment it was assumed that pigs in barren environments and/or without the sedative effects of the herbal compound would be subjected to a more stressful environment and this was considered a chronic stress scenario to test the validity of CgA and hair cortisol as indicators. Overall, the results showed lower levels of both hair cortisol and salivary CgA in the animals supplied with enrichment material and/or sedafit. Each indicator is discussed separately as follows.

4.1. Hair cortisol

The present results are in agreement with a growing number of studies linking hair cortisol and HPA axis activity in different species (Stalder and Kirschbaum, 2012). To the knowledge of the authors, only a limited number of studies have focused on hair cortisol in pigs: Casal *et al.* (2014) in growing pigs, Martelli *et al.* (2014) in transgenic pigs and their relatives, and Bacci *et al.* (2014) in sows. Cortisol levels found in the present study (Table 1, Figure 2), when the animals were aged 16 weeks, are slightly lower to those reported at the age of 17 weeks in a previous experiment using growing pigs subjected to a weekly remixing procedure (Casal *et al.*, 2014) and, somehow, taking into consideration the different production stage, to levels in sows by Bacci *et al.* (2014). Furthermore, whilst hair cortisol concentration of the pigs belonging to this previous experiment on the effects of weekly remixing increased significantly throughout time (Casal *et al.*, 2014), in the present experiment hair cortisol concentration of pigs supplied with the herbal compound, enrichment material and both decreased at the age of 24 weeks (T2), when compared to the initial concentration. In contrast, for the control group, a significant (but smaller, as compared to treatment

groups) decrease was observed between T0 and T1, and levels increased again at T2. Our hypothesis is that the lower levels of cortisol detected in the treatment groups may indicate a lower level of stress. This decrease could be associated with an age effect, but in this case a decrease would have been expected for the control group, and this was not the case for T2 levels.

On the other hand, our hypothesis to explain why these differences were not reported at 20 weeks of age (T1) should be partly explained by the shaving procedure. Before each sample, hair was shaved to ensure a similar length (and age) of the hair comprised between two consecutive samples. However, part of the hair analysed was not belonging to the in-between samples' periods, since the hair under the skin was not considered. It is known that the deep of the hair in growing pigs is about 3-4 mm (Mowafi and Cassens 1976). As a consequence, assuming that cortisol can be incorporated into growing hair cells via passive diffusion (Stalder and Kirschbaum, 2012), 3-4 mm of hair remained in the skin and were analysed in the next period. According to Bacci *et al.* (2014), the proportion of hair remaining in the skin belongs to 15 days approximately. Thus, the sample taken at the age of 20 weeks, was composed by 3-4 mm of hair probably containing cortisol information from approximately 15 days before starting the experiment (< week 16 of age) masking the effect of the treatments. Furthermore, the fact that in the previous experiment levels were found to increase over time when subjecting pigs to a chronic stressor, like weekly remixing, adds further support to the finding that levels of cortisol in hair may be indicative of exposure to chronic stressors.

No correlations between salivary and hair cortisol were reported in this study. Sauvé *et al.* (2007) did not report a significant correlation between hair cortisol and salivary cortisol in humans (r =0.31, P = 0.12) with a single time-point sample either. On the contrary, van Holland (2012) found a moderate correlation (r=0.41, p=0.03) in humans, but taking six samples per day for three days. D'Anna-Hernandez *et al.* (2011) reported correlations when samples taken over a long period of time were considered. Therefore, more saliva samples over a longer period may be required and are strongly recommended to study the correlation between saliva and hair in pigs.

4.2. Chromogranin A

Salivary CgA has been reported as being a good acute stress indicator in humans (Kanamaru *et al.*, 2006) and in pigs (Escribano *et al.*, 2013 and 2015; Ott *et al.*, 2014). Although no studies regarding the relation between CgA and chronic stress were found, the authors hypothesize that the storage described in the secretory granules of the neuroendocrine tissues may produce increased CgA levels in chronically stressed animals. Thus, in this experiment, the differences between treatments may

be explained because the groups supplied with either enrichment material or the herbal compost produced and stored less CgA, as compared with the control group. Furthermore, steady levels of CgA in the control group may indicate again, as for cortisol, that the decrease over time may not be associated with age itself, but rather with the differential stress conditions of the two treatments.

The levels of CgA reported in this experiment are similar to those previously obtained in pigs by Escribano *et al.* (2013), when firstly presenting CgA as a stress indicator in this species. These authors reported higher CgA concentrations after acute stress (immobilization). Similar results were observed 30 min after isolation and 30 min after regrouping the isolated animals (Escribano *et al.*, 2015). Increased CgA levels were also reported after feed deprivation, but surprisingly, not after mixing animals (Ott *et al.*, 2014). A hypothesis to explain why CgA levels can be affected by acute and chronic stress situations could be that while in acute stress there is a punctual increased production and release of CgA, in chronically stressed animals, the basal levels of CgA may be increased due to a higher number of molecules stored in the vesicles of neuroendocrine tissues and secretory granules of acinar cells. Furthermore, the correlation observed between hair cortisol and CgA, although moderate, adds further support to CgA as a chronic stress indicator because of the more widely reported increase of hair cortisol under chronic stress situations.

A moderate correlation, higher than the reported in the present study, was described between CgA and salivary cortisol by Escribano *et al.* (2013) and Ott *et al.* (2014). On the other hand, Escribano *et al.* (2015), in a different experimental model, did not find a correlation between either analytes. In the studies reporting higher correlations, pigs were subjected to a punctual stressor in order to produce an acute stress response, while in the present study stress was not induced to the animals. Thus, we suggest that a stronger correlation may be reported when an acute stress response is triggered than when assessing baseline levels.

4.3. Salivary cortisol and TNF-α

The authors expected an effect of environmental enrichment and herbal compound on both salivary cortisol and TNF-*α,* which was not found. Salivary cortisol was chosen as biomarker of HPA response to be compared with hair levels, and inconsistent results were found. No differences were observed at the beginning or at the end of the experiment, but after one month of starting the treatment, lower salivary cortisol levels were observed in the animals raised in an enriched environment, as compared with the animals raised in a barren environment. Whereas de Leeuw *et al.* (2004) and Merlot *et al.* (2012) reported lower levels of salivary cortisol on enriched pigs, de Jong *et al.* (1998)

and de Groot *et al.* (2000) found higher levels, and Morrison *et al.* (2007) did not reported differences between housing environments.

Although serum TNF–α was expected to be lower in the treatment groups, no significant differences were reported in this experiment. Paik *et al.* (2000) did not found changes in students under exam conditions either. On the contrary, divergent results have been reported in relation to acute stress. Tuchscherer *et al.* (2010) reported decreased TNF-α levels in isolated neonatal piglets. Similar results were observed by Pearce *et al.* (2013), who found lower levels in pigs under heat stress. On the contrary, Ciepielewski (2013) reported higher levels in pigs during prolonged restraint. More studies are needed to further understand the response of salivary cortisol and serum TNF-α in relation to chronic stress, but our hypothesis is that both salivary cortisol and serum TNF-α could be better indicators for acute stress response, as suggested by previous studies (Connor *et al.*, 2005; Mormède *et al.*, 2007), rather than for chronic stress situations.

5. CONCLUSIONS

Hair cortisol and chromogranin A levels were found to be lower under expected less stressful conditions (*i.e.* when pigs were provided with enrichment material and herbal compound). Thus, both chomogranin A and hair cortisol may be useful tools to detect chronic stress. Furthermore, chromogranin A appears to be more sensitive, since changes could be detected early than in hair cortisol. More studies are required to better understand the role of chromogranin A and hair cortisol in relation to chronic stress response and, therefore, recommending its routine use as biomarkers of chronic stress conditions.

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Chapter 3

Influence of enrichment material and herbal compounds in the behaviour and performance of growing pigs

Based on a paper submitted to Applied Animal Behaviour

ABSTRACT

Pigs reared in barren conditions are exposed to many different stressors, compromising their welfare and well-being and producing physiological and behavioural changes. The aim of this study was to assess the effect of environmental enrichment (EE) consisting in natural hemp ropes, sawdust and rubber balls, and a herbal compound (HC) of *Valeriana officinalis* and *Passiflora incarnata* on the behaviour and performance of growing pigs. Fifty-six pigs were used to assess four different treatments divided in two pens of seven animals per treatment (14 pigs/treatment). The treatments tested were: a) pigs reared with EE, b) pigs supplemented with HC, c) pigs provided with both EE and HC, and d) control group (CG, neither EE nor HC). Body weight and lesions were measured at 18, 20, 22 and 24 weeks of age. Body weight was also recorded before starting the experiments (week 15) and lesions the day after mixing. Weekly instantaneous scan and continuous focal sampling were used to record behavioural patterns of activity, social interactions and abnormal behaviours. Three novel tests were carried out at 16, 19 and 23 weeks of age. Body weight at the end of the experiment was found to be significantly lower for the pigs reared in the control group compared to the other treatments ($p=0.0009$). Furthermore, pigs reared with EE presented less stereotypies ($p=0.016$) and redirected behaviour (0.0188) but more exploratory behaviour ($p=0.008$). On the other hand, pigs supplemented with HC presented less social interactions ($p=0.048$), a trend to present less negative social behaviour ($p=0.09$) and less skin lesions ($P=0.0433$) than pigs not supplemented. Finally no remarkable differences were reported in any of the three novel tests. Thus, both EE and HC positively influenced animal welfare and performance of growing pigs in the present experiment.

1. INTRODUCTION

Most of the European pig production is based on intensive farming. Intensification mainly aims at optimising production costs, but it may present some detrimental implications on animal welfare grounds. The interest in animal welfare has increased during the last decades (Sandøe and Simonsen, 1992), driving to a higher demand for more "animal friendly" systems. Although the concept of animal welfare allows different interpretations (Mason and Mendl, 1993), it is generally accepted that stress negatively influences animal welfare (Veissier and Boissy, 2007). Stress is often defined as a threat to the homeostasis of the organism (Chrousos and Gold, 1992) that activates a broad range of complex physiological, behavioural and neurological changes (Chrousos, 2009). If this response is prolonged, inadequate, excessive, or there is a failure to cope with the stressor it may produce adverse consequences on the physiology of the organism producing immunological, metabolic, reproductive and behavioural alterations (Möstl and Palme, 2002).

Growing pigs in barren environments are exposed to many stressful factors, with the subsequent negative implications for their welfare and health (Ruis *et al.*, 1997). In fact, a barren environment has been suggested as an important stress factor by itself because pigs present limitations to express their foraging species-specific behaviour, leading to frustration (for a review see: van de Weerd and Day, 2009). Environmental enrichment motivates the exploratory behaviour, which is considered a need for pig's welfare (Studnitz *et al.*, 2007), so to provide enrichment material in pigs is one of the strategies to reduce stress.

Another described strategy to reduce stress is by means of plants with sedative and tranquilizer properties such as *Valeriana officinalis* and *Passiflora incarnata* (Murphy *et al.*, 2010; Peeters *et al.*, 2004; Soulimani *et al.*, 1997). The mechanism involved in the sedative and anxiolytic properties of *Valerian officinalis* seems to be mediated by the interaction of Valerian Acid with the γ-Amino butyric acid receptors type A ($GABA_A$). The stimulation of $GABA_A$, opens the permeability of chloride channels, producing neural inhibition (Khom *et al.*, 2007; Murphy *et al.*, 2010). Moreover, different bioactive compounds have been detected in *Passiflora incarnata* such as flavonoids, maltol, cynogenic glycosides and indole alkaloids, without a consensus regarding the most important one for the sedative properties. Flavonoids, which are beyond the most studied components, have a similar effect than Valerian Acid, increasing the membrane permeability by means of the modulation of $GABA_A$ (For a review see: Miroddi *et al.*, 2013).

The aim of this work was to study the effect of environmental enrichment by means of natural hemp ropes, sawdust and rubber balls, and the effect of supplementing the diet with a herbal compound

containing Valerian (*Valeriana officinalis)* and May pop (*Passiflora incarnata)* on the behaviour and other animal-based measures (body weight and skin lesions) in growing pigs. The hypotheses to be tested was that pigs grown in an enriched environment and/or supplemented with these herbal compounds should be less stressed and present less abnormal behaviour, less skin lesions and would grow up more than pigs kept in barren conditions and/or without this supplement in the diet.

2. MATERIALS AND METHODS

2.1. Animals and housing conditions

Fifty-six entire males [(Landrace x Large white) x Pietrain] were used in this experiment. Pigs arrived to the facilities at the age of ten weeks with a mean weight of 25 kg, and were kept together before starting the experiment. At the age of 16 weeks and a mean weight of 49.8 kg, pigs were randomly distributed in two different modules. Each module had four pens of 13.67 m^2, two on the left and two on the right separated by a corridor. The stocking density per pen was 1.95 m^2/pig. Food and water were supplied *ad libitum* by two hoppers and a single drinker. This distribution allowed to test four treatments in two replicates. Since the aim of this experiment was to study the effect of environmental enrichment and supplementation of a Herbal compound, the treatments assessed were as follows: 1) pigs supplemented with both environmental enrichment and herbal compound (named as EEHC pigs from this point), 2) pigs supplemented with environmental enrichment (EE), 3) pigs supplemented with the herbal compound (HC) and 4) control group (CG), consisting in pigs kept in a barren environment and without herbal compound supplementation. The enrichment material consisted in natural hemp ropes, sawdust and rubber balls, all provided at the same time, during all the experiment. In order to support the sawdust, 1/3 of the slats in the pens supplied with it were partially covered with polypropylene sticks (Click-in®, Rotenca, Agramunt, Spain), and new sawdust was added every two days. A single rubber ball with a diameter of 15 cm was provided in each enriched pen. Two hemp ropes were hanged in the walls of EE and EEHC pens, and were substituted when the length of the rope after its use by the pigs was lower than 30 cm. The herbal compound used (Sedafit ESC, Phytosynthèse, Saint-Bonnet de Rochefort, France) contains Valerian (*Valeriana officinalis*) and Maypop (*Passiflora incarnata*). Weight was recorded at the age of 15 (before starting the experiment), 18, 20, 22 and 24 weeks using a cage with a scale. Skin lesions were assessed the day after mixing the animals and at 18, 20, 22 and 24 weeks of age taking advantage of the restrain provided by the cage during the weighing procedure. The total amount of lesions in each one of the five regions defined in the Welfare Quality® (2009) were recorded in addition to score the animal as

0, 1 and 2 according to the protocol. Furthermore, blood, saliva and hair samples were taken before starting the experiment (15 weeks of age), at the middle (20 weeks of age) and at the end (24 weeks of age) for cortisol, chromogranin A and TNF-α quantification as part of a broader study. All the procedures carried during this experiment were approved by the IRTA ethical committee.

2.2. Behavioural observations

Instantaneous scan sampling and continuous focal sampling as described in Martin and Bateson (1993) were used to record behaviour. Once per week, during nine weeks, direct observations of each module (4 pens) in sessions of two consecutive hours (11:00- 13:00) were carried out by a trained observer. The observations were performed in a specific order to avoid possible differences of time between groups. Scan samplings were taken every ten minute intervals, and focal samplings elapsing six minutes per pen were recorded between two consecutive scan samplings. Thus, each observation day provided a total of 12 scans per animal and 18 minutes of focal sampling per pen, divided in three periods of six minutes. The behaviours observed according to each observational methodology are summarised in Table 1. Exploratory behaviour was analysed grouping the interaction with the pen and the interaction with the enrichment material. Twenty minutes before starting the observations, the observer entered into the module and walked during ten minutes among the corridor with the aim of allowing the pigs to get used to his presence. Then, the observer moved to the centre of the module, and stayed there for another ten minutes before starting the observations. Observations were done from the centre of the module with full visual contact to all the pens.

Table 1: Ethogram used for both scan and focal samplings

Specific category	Definition
SCAN SAMPLING	
Activity	
Standing inactive	Pig is upright on all four legs, neither moving forward or backward
Walking	Pig is upright on all four legs, and moves in the pen
Sitting inactive	Pig is upright on two front legs, and hindquarter
Lying	Pig is recumbent on its belly or side
Behaviour	
Eat/Drink	Head or snout over bowl or trough
Interaction with the pen*	Licking, chewing, nosing or sniffing unanimated objects from the pen, excluding enriched material
Interaction with EM*	Interaction with sawdust, ropes or balls
Social Interaction	Head or snout in contact with another pig (includes any positive or negative social behaviour)
Other behaviours	Behaviours different than those previously described
FOCAL SAMPLING	
Positive social behaviour	Head or snout in contact with another pig. The receiver does not react negatively
Stereotypies	Stereotypic behaviour, basically animal rubs the bars of the crate with the mouth, and other stereotypic behaviours
Redirected behaviour	Vertical rub movements with the snout to the belly of a pen-mate (belly nosing), bite or suck the ear of a pen-mate (ear biting) and bite or suck the tail of a pen-mate (tail biting)
Negative social behaviour	Aggression, fights to another pig with a negative response from the receiver
Sexual behaviour	Sexual behaviour, one animal mounting or trying to mount another pig
Other behaviours	Any other behaviour

*Exploratory behaviour was analysed grouping the Interaction with the pen and the interaction with the enrichment material (EM).

2.3. Novel tests

At 16 weeks of age pigs were subjected to a novel object test in a novel environment. The novel object was a traffic cone filled with cement to avoid the potential movement produced by pigs when contacting it. A second novel test was performed at 19 weeks of age, and consisted in a PVC ball of 55 cm (Fitball 55cm Domyos, Decathlon, Villeneuve-d'Ascq, France) suspended from the ceiling at 30 cm from the floor. Finally, a third novel test was performed with a human-figure at 23 weeks of age; the test consisted in a caretaker dressed in a white overall with cowl and with a disposable mask.

All the novel test were performed in the same room. The test room (Figure 1) was 31.5 m^2 (4.7m x 6.7 m), and was at a distance of 6.4 m from one of the modules and at 20.6 m of the other module. In order to balance the distance, a short walk was done with the animals of both treatments before starting the test so that the final time taken to reach the test pen was the same (close to one minute). All pigs were conducted to the test room by the same caretaker. The novel objects were placed straight to the entrance at 3.5 m. Linear and concentric lines were drawn on the floor every meter for activity analysis. Observations for behaviour analyses were done for up to five minutes through a door window. After five minutes, if the animal did not touch the object, pigs were conducted to their home pens. All the observations were carried out by the same observer. The parameters observed in the novel test were time to cross the concentric line of two meters and one meter to the object, latency to contact the object, total lines crossed, number of extra contacts, number of vocalizations, reluctance to enter into the room and excretory behaviour.

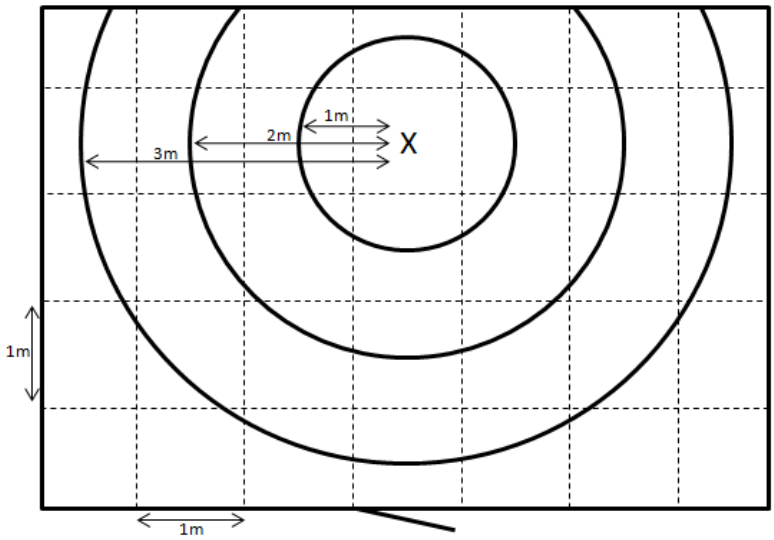

Figure 1: Schematic representation of the novel test room with concentric lines every meter and lines drawn in the floor for activity evaluation. X represents the place where the object was placed.

2.4. Statistical analysis

All the statistical analyses were conducted using Statistical Analysis System (SAS version 9.2; SAS institute Inc., Cary, NC; USA). Significance was established at $p<0.05$ for all the analyses, while a tendency was considered between $p>0.05$ and $p<0.1$. Results are presented as mean ± standard error (SEM) unless otherwise indicated. Shapiro-wilk test (with PROC UNIVARIATE in SAS) was used to examine the normality of distributions.

Weight presented a parametric distribution, and it was analysed using generalized linear mixed model (MIXED procedure and the covariance matrix AR(1) in SAS). Use or not of herbal compound and environmental enrichment, sampled day and their interactions were considered fixed effects, while pen was considered as a random effect. Furthermore, weight before starting the experiment was considered as a covariate after observing no differences between treatments. Least square means of fixed effects (Lsmeans) with tukey adjustment were used for comparisons when analysis of variance indicated differences ($p<0.05$).

Behavioural data from scan samplings were analysed as a percentage of the scans of each category in relation with the total number of scans per day, while data from focal sampling were analysed as the total number of behaviours observed in each category per pen and day. Behavioural data from scan samplings (except for activity) and focal samplings were analysed by means of non-parametric generalized linear models (GENMOD procedure) with repeated measurements and the covariance matrix AR(1). A Poisson or a negative binomial distribution was used based on the deviance (Cameron and Trivedi, 1998). Use or not of enrichment material and herbal compound, sampled day and their interactions were considered as fixed effects. The experimental unit was the pen. Interactions were analysed using Lsmeans with tukey adjustment when analysis of variance indicated differences ($p<0.05$). Behavioural data of activity taken with scan sampling were analysed by means of a multinomial model using as well GENMOD procedure with repeated measurements and the covariance matrix AR(1). The treatment was considered the fixed effect, being the pen the experimental unit.

Skin lesions were analysed with a negative binomial distribution using the GENMOD procedure. Pen was considered as experimental unit, and use or not of herbal compound and environmental enrichment, sampled day and their interactions were considered fixed effects. Lesions before starting the experiment were considered as a covariate. Interactions were analysed using Lsmeans with tukey adjustment.

Novel tests were analysed individually by means of non-parametric generalized linear models (GENMOD procedure). A Poisson or a negative binomial distribution was used based on the deviance (Cameron and Trivedi, 1998). Use or not of enrichment material and herbal compounds and the interaction were considered as fixed effects. Lsmeans with tukey adjustment were used for comparisons when analysis of variance indicated differences ($p<0.05$).

3. RESULTS

3.1. Body weight

Body weight at 16 weeks was not significantly different between treatments (50.22 ± 1.17; 49.61± 1.05 ; 49.64± 1.26 ; 49.81± 1.22, for CG, HC, EE and EEHC, respectively) and was used as a covariable. Table 2 summarises the results of the interaction between treatments throughout time (P=0.0009). Pigs in the control group presented a significantly lower weight at 24 weeks of age compared to EE, HC and EEHC pigs.

Table 2: Live weight of pigs of the different treatments throughout the experimental time

	ALL	CG	HC	EE	EE HC	SEM
N	55	14	14	14	13	
18 weeks	64.82	64.46	65.24	65.49	64.09	0.69
20 weeks	79.02	77.64	78.28	79.27	80.86	0.69
22 weeks	91.94	89.43a	90.78[a]	93.63[b]	93.90[b]	0.69
24 weeks	109.82	104.5a	110.35[b]	111.79[b]	112.35[b]	0.69

SEM= standard error of least square means.
Least Square means in a row with different superscripts differ significantly at p<0.05. EM= pigs grown in an enriched environment, HC= pigs supplemented with a herbal compound with sedative properties, EM HC= pigs grown in an enriched environment and supplemented with herbal compound, and CG=pigs with neither enrichment material nor herbal compound (control group).

3.2. Skin lesions

Less skin lesions were observed in the groups of pigs supplemented with the HC compared with those not supplemented (mean skin lesions per day: 7.12± 0.55 vs 5.32 ±0.41; p=0.043).

3.3. Behavioural observations

No time effect was found on any of the behavioural parameters recorded, thus, time was removed to simplify comparisons between treatments. With regards to activity patterns, EE pigs stayed less time lying or sitting compared with CG, HC and EEHC (p=0.019) (Figure 2). The effect of environmental enrichment and supplementation of herbal compound on the other behaviours recorded is presented in Table 3. Pigs reared in enriched conditions (EE and EEHC) performed less stereotypies (p=0.016) and less redirected behaviour (p=0.0188) than pigs reared in barren conditions (CG and HC). Furthermore, EE and EEHC pigs presented more exploratory behaviour compared with CG and HC pigs (p=0.008). Furthermore, EE and EEHC pigs tended to have less positive interactions than GC and HC pigs (p=0.089), and showed a tendency to present more sexual behaviour (p=0.06). On the other hand, animals supplemented with the herbal compound (HC and EEHC) showed less social interactions (p=0.048) than pigs not supplemented (EE and CG), although no significant differences but a trend was reported during the focal samplings, with lower negative social behaviours (p=0.09) in HC and EEHC compared with EE and CG.

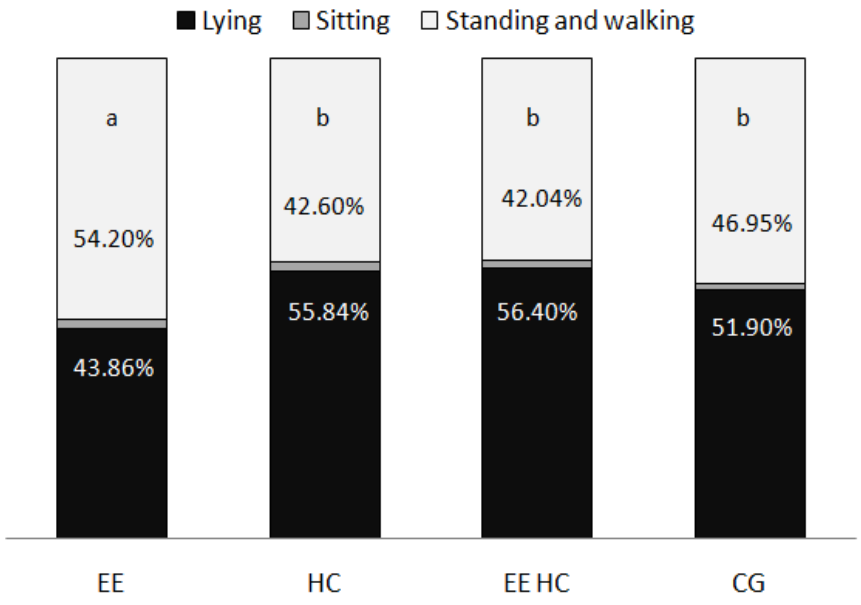

Figure 2: Percentage of activity patterns of pigs subjected to the different treatments: pigs grown in an enriched environment (EE), pigs supplemented with a herbal compound with sedative properties (HC), pigs grown in an enriched environment and supplemented with herbal compound (EEHC), and pigs with neither enrichment material nor herbal compound (CG). Different letters represent significant differences between groups (p<0.05)

Table 3: Effect of environmental enrichment and herbal compound supplementation on behaviour of pigs during the whole study

	Housing system[b]		*Herbal compound*[c]			*p-value*	
Behaviour	*BE*	*EE*	*no*	*yes*	*SEM*[a]	*HS*[b]	*HC*[c]
Scans							
Exploration	4.17	12.13	8.87	7.42	0.49	**	*ns*
EM[d] interaction	-	-	11.42	7.22	0.45	-	*ns*
Social interactions	4.06	3.85	5.23	2.68	0.25	*ns*	*
Focals							
Positive social behaviour	5.84	4.31	4.56	5.59	0.39	0.09	ns
Stereotypies	4.88	1.22	2.97	3.13	0.43	*	ns
Redirected behaviour	0.75	0.28	0.50	0.53	0.09	*	ns
Negative social behaviour	7.25	5.84	7.84	5.25	0.77	ns	0.1
Sexual behaviour	0.38	1.19	0.78	0.78	0.17	0.06	ns

[a] SME: Standard error of the mean

[b] Housing system (HS) BE=Barren environment/ EE= enriched environment.

[c] Herbal compound (HC) yes=supplied with herbal compound/ no= not supplied.

[d] EM= Enrichment material consisting in sawdust, ropes and balls

p-values are represented by *P<0.05; **p<0.01; ***p<0.001; tendency p<0.1 and ns not significant. Behaviours from scan samplings are expressed as a % of total scans per pen while behaviours from focal samplings are expressed as mean occurrence of the behaviours per pen. Time was removed to simplify comparisons between treatments.

Interaction HS*HC was not significant for any parameter.

3.4. Novel test

No significant differences were observed in neither the novel test performed with a cone (16 weeks of age), nor the novel test performed with the human-figure (23 weeks of age) in any of the parameters observed. When a PVC ball was used (19 weeks of age), pigs maintained in barren conditions (CG, HC) crossed the two-meter line before than pigs grown in enriched conditions (EE, EEHC; p=0.012). Furthermore, the activity of the pigs during the novel test tended to be higher for the pigs reared in barren conditions (CG, HC) at 16 weeks (p=0.10) and at 23 weeks of age (p=0.07) in comparison to EE and EEHC.

4. DISCUSSION

This study adds support to the hypotheses that enrichment material and some bioactive constituents from herbal compounds have a positive effect in the welfare and performance of growing pigs. Previous studies have also reported the beneficial effects of environmental enrichment in pigs (for a review see: Van de Weerd and Day, 2009), while, up to the knowledge of the authors, the effect of herbal compounds on welfare in pigs has only been published twice (Peeters *et al.*, 2004, 2006).

In the present study EE, HC and EEHC pigs were found to present a significant increase in body weight compared with the control group at 22 and 24 weeks of age. This finding could indicate that environmental enrichment and herbal compounds can improve the growth of pigs since they were slaughtered at the same age. Furthermore, these results are supported by the work of Beattie and collaborators (2000) who described a higher body weight, feed intake, food conversion and growth rate in pigs from a similar age raised in an enriched environment (weights comprised between 15 and 21 weeks of age). Moreover, increased growth rates have been previously related with an increased food intake in pigs reared with straw (Lyons *et al.*, 1995). Although no straw was used in the present experiment, sawdust, which is a similar material even preferred to straw in some studies (Beattie *et al.*, 1998), was provided to the enriched pens together with point-source objects. Literature on the effects of point-source objects on performance present more inconsistent results compared to the use of straw (Van de Weerd and Day, 2009). An improved animal growth was reported when providing car tires and both mineral block and a teeter-totter device (Schaefer *et al.*, 1990). However, Hills *et al.* (1998) found an increased average daily gain and food:gain ratio in the animals exposed to "toys", but only in the finishing stage and for one of the genetics tested (EXP-94). Two main hypotheses are proposed to explain the increased body weight reported in the pens with enrichment material. First of all, according to Lyons and collaborators (1995), when enrichment is provided pigs are more active and present a higher exploratory behaviour, which facilitates the contacts with the feeder and consequently, may increase the feed intake. Indeed, as it is highlighted below, an increase in exploratory behaviour was reported in the present experiment in pigs reared in enriched environments. The second hypothesis, is related with the reduction of anxiety and stress reported in the animals reared in enriched conditions (Schaefer *et al.*, 1990), which have been described to reduce cortisol and catecholamines, both with a catabolic activity (Mormède *et al.*, 2007). Along that line, the fact that in the present experiment general activity was also increased by environmental enrichment without a negative effect on weight, could indicate that stressed animals are less efficient in food conversion because of the elevated levels of cortisol (De Jonge *et al.*, 1996).

To support this hypothesis, differences in some stress indicators such as cortisol and chromogranin A were reported in the same pigs as a part of a broader investigation (Casal *et al.*, 2014).

No studies were found about the use of herbal compounds related with body weight, but the sedative effect produced by the active components of the plants may explain this increase since a reduction of the activity could be expected. As discussed below, general activity was not reduced when herbal compound was supplied in the present study. However, the reduced number of skin lesions observed in the animals supplemented with the herbal compound together with the trend to a reduced number of social negative behaviour, would support the hypothesis that herbal compounds may have a sedative effect, reducing the number of aggressions and probably diverting energy from negative social behaviour to growth. These results are in discordance with those reported by Peeters *et al.* (2006) who found more lesions in the shoulder and loin of pigs supplemented with the herbal compound, although this finding was not expected by the authors. In both experiments, the product used was the same (Sedafit) but while in Peeters *et al.* (2006) the herbal compound was dissolved in the water, in the present study it was mixed with the food. Furthermore, in Peeters *et al.* (2006) the evaluation of the lesions was done at the slaughterhouse line after different acute stressors such as fasting for 18 hours, mixing and transporting the pigs to the abattoir, while in the present study lesions were assessed periodically on farm.

On the other hand, environmental enrichment did not have an effect on neither negative interactions nor skin lesions. Although some studies reported reduced levels of aggression in enriched environments, literature shows very controversial results (Van de Weerd and Day, 2009). The increased amount of aggressions in barren pens can be a consequence of the increased levels of redirected behaviour to the pen mates (Beattie *et al.*, 1996). Nevertheless, deficient enrichment materials can increase the aggressions by enhancing the competition, which can produce more negative agonistic behaviour, producing frustration and/or aggression (Docking *et al.*, 2008). Furthermore, more aggressions have also been previously related with a bad location of the enrichment material, since it can cause disruption to other pen mates (Van de Weerd and Day, 2009). Thus, type and quality of the material, location, novelty effect, and number of objects should be taken into account so that the enrichment becomes meaningful and available to pigs, properly enhances exploratory behaviour and obtains the expected beneficial effect on their welfare.

The present experiment observed also a significant increase in the exploratory behaviour of pigs reared in enriched environments compared with pigs reared in barren environments in agreement with previous studies (Beattie *et al.*, 2000). Pigs reared with environmental enrichment have been

said to be more stimulated (Van De Weerd *et al.*, 2003). The manipulation of the substrates provided as enrichment fulfils better the pig exploratory needs, since pigs have a preference for chewable, destructible, rootable and deformable materials (Bolhuis *et al.*, 2005). The present study confirms that properly manipulative materials are those capable of increasing exploratory behaviour in pigs, since no significant differences in exploration were found when only considering interactions with pen structures. Exploratory behaviour is considered as a need for pig's welfare; as a consequence, pigs reared in barren conditions are more susceptible to present behavioural problems such as stereotypies and redirected behaviours to other pen mates in an attempt to satisfy their exploratory needs (Studnitz *et al.*, 2007). Stereotypies are considered a repetitive, invariable and functionless behaviour closely related with stress and poor animal welfare (Mason *et al.*, 2007). The present study reported less stereotypies in pigs reared in enriched conditions compared with pigs reared in barren conditions in agreement with previous studies (e.g. Chamove, 1989). Furthermore, redirected behaviour to other pen mates such as tail biting, ear chewing or belly-nosing was also significantly lower in the animals reared in an enriched environment as previously reported (De Jong *et al.*, 1998; Jensen *et al.*, 2010; Petersen *et al.*, 1995). Moreover, pigs reared in enriched environments spent more time active than pigs reared in barren conditions in agreement with previous results (Beattie *et al.*, 1995; Bolhuis *et al.*, 2005; Bracke and Spoolder, 2008). However, the activity of EEHC was not enhanced, and presented similar levels to HC and CG. A possible hypothesis to explain the difference between EE and EEHC is somehow related with the sedative properties of the herbal compound which can counteract a hypothetical increase of the activity produced by the enriched material. Nevertheless, HC pigs did not differ from CG with regards to activity patterns. Thus, more research is needed to better clarify the potential reduction on the activity modulated by the herbal compounds. In fact, if herbal compound are able to produce a reduction of the activity in pigs, this reduction must not be confounded by the lack of motivation produced by barren environments or inappropriate enrichment material.

Novel object tests are commonly used to assess fear and anxiety (Dalmau *et al.*, 2009). A latency to touch a novel object and less time spent investigating the stimulus have been related with higher levels of anxiety or fear (Murphy *et al.*, 2014). On the other hand, an increased excitability, a quicker approach and longer interactions in front of a novel object have been previously described in pigs reared in barren conditions compared with pigs reared in enriched conditions (Bracke and Spoolder, 2008; Grandin *et al.*, 1987; Pearce and Paterson, 1993; Stolba and Wood-Gush, 1980). Accordingly, pigs in barren conditions crossed quicker than pigs reared with enrichment material the two meters line from the door to the novel object test when a PVC ball was used. Stolba and Wood-Gush (1980)

argued that pigs usually kept in barren environments could present a stronger motivation to explore what could support the higher latency of enriched pigs to cross the two meters line compared to the barren pigs. However, no other significant effect was found for this or the other novelty tests used.

5. CONCLUSIONS

The results of the present work highlight a positive effect of rearing pigs in an enriched environment and/or supplementing the diet with a herbal compound containing *Valeriana officinalis* and *Passiflora incarnata*. Environmental enrichment reduced some abnormal behaviours such as stereotypies and redirected behaviour, while increased exploratory behaviour and the activity of the pigs. On the other hand, pigs supplemented with the herbal compound presented less negative interactions and less skin injuries. Furthermore, pigs reared in enriched conditions, supplemented with a herbal compound or with both treatments at the same time presented higher body weight compared to the control pigs.

6. ACKNOWLEDGEMENTS

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Chapter 4

Effect of environmental enrichment and herbal compounds on pig carcass, meat quality traits, and consumers' acceptability and preference

Based on a paper submitted to Meat Sicence

ABSTRACT

The aim of the present study was to evaluate consumer's acceptability, and preference, and meat and carcass quality of pork reared with two strategies aiming to reduce the stress on farm. The factors analysed in a 2x2 factorial design were the supplementation of an herbal compound containing *Passiflora incarnata* and *Valeriana officinalis* both with sedative properties, and the effects of environmental enrichment by the provision of hemp ropes, sawdust and rubber balls. A total of 56 pigs were divided in four treatments in two pens of seven pigs per treatment. The four treatments tested were: a) pigs supplemented with both enrichment material and herbal compound (EEHC), b) pigs supplemented with enrichment material (EE), c) pigs supplemented with the herbal compound (HC) and d) control group (CG). Carcass and meat traits such as live and carcass weight, fat depth, muscle thickness, lean content, carcass and loin length, pH, colour, intramuscular fat, electrical conductivity, marbling and drip loss were measured from each pig. Consumer's acceptability and preference were analysed by means of a sensory test, and a conjoint analysis respectively with a total of 110 consumers. Previous to slaughter, pigs from control group presented lower live weight compared with pigs from EEHC, EE and HC ($p=0.0009$), but no other differences in carcass and meat quality were reported. Furthermore, although the acceptance was the same for all the groups in the sensory analysis, consumers preferred the systems aiming to increase the welfare of the pigs. The most important factor was the production system, with preference for systems with improvements for the animal welfare, followed by the feeding system, with preference for systems supplemented with natural herbs with relaxing properties while the least important factor considered was the price. However, price was the most important factor for a segment of consumers, with a clear preference for lower prices. The present results suggest that improvements in animal welfare could be appreciated by particular segments of consumers.

1. INTRODUCTION

By the middle of the 20th century, most of the industrialized countries augmented considerably the agricultural production by means of implementing the so called intensive production systems. Pigs reared in intensive conditions are faced to many different stressors during their lives which may negatively impact on their welfare (Bolhuis *et al.*, 2005). Due to this intensification, ethics and animal welfare concerns have arisen, especially since the publication of Animal Machines by Ruth Harrison (1964). Furthermore, there is the general perception that besides the well-being of the animals, increasing the welfare has also a positive impact on the food safety, quality and healthiness of consumers (Harper and Henson, 2001). Consequently, the demand for more welfare-friendly food products augmented considerably during the last years (Napolitano *et al.*, 2010).

Different intrinsic characteristics of pork meat such as colour, taste, texture, and odour, and some extrinsic characteristics such as price, food safety, origin and information on animal production may influence consumer behaviour (García-Torres *et al.*, 2016; Grunert *et al.*, 2004). These different attributes are not perceived with the same importance by different consumers. Thus, not all consumers have the same beliefs, attitudes and behaviours, toward the same product (Verbeke *et al.*, 1999). Animal welfare appears to be among the most important factors to consider for some pork meat consumers (Meuwissen *et al.*, 2007). However, although most consumers report a certain willingness to pay more for products with an animal welfare guarantee, this is not always translated into a real consumption behaviour (Harper and Henson, 2001). Access to information regarding the welfare issues and trust in the information provided by the labels have been suggested to be among the most important factors for those consumers aiming to purchase animal-friendly products (Toma *et al.*, 2012). Along that lines, most of the consumers indicate a lack of information about production systems and market transparency (Gracia *et al.*, 2009). Besides, most of them are not willing to know specific details of how animals are treated, but they would like to have more transparent, enforceable, and traceable information with a labelling system for animal welfare products (Frewer *et al.*, 2005). On the other hand, there is the general perception that the interest of the industry in animal welfare is merely economic (Kendall *et al.*, 2006). In this context, a labelling system which guarantees an optimal animal welfare in farming practices is seen as one of the best alternatives.

Several strategies can be implemented when attempting to increase welfare standards in pig production systems. This study has focused on two management strategies: introduction of environmental enrichment materials and provision of a sedative herbal compound. Both strategies have been previously used as stress reducers in pigs (Averós *et al.*, 2010; Peeters *et al.*, 2006).

Regarding the environmental enrichment, it is mandatory in the European Union (Council directive 2008/120/EC), because rearing pigs in barren conditions may produce a broad range of behavioural and physiological problems (for a review see: van de Weerd and Day, 2009). Exploratory behaviour is considered a need for pig welfare (Studnitz *et al.*, 2007), thus, the provision of enrichment material enhances some species-specific behaviours such as foraging reducing in turn the frustration of the pigs. In relation with the herbal compound, the main active components of the product used (sedafit) were *Valeriana officinalis* and *Passiflora incarnata*. Both plants have different bioactive compounds with sedative or relaxing properties (Dhawan *et al.*, 2001; Murphy *et al.*, 2014).

The aims of the present study were: (1) to evaluate carcass and meat quality characteristics of pork meat produced using two strategies aiming to reduce the stress on farm: supplementation of an herbal compound with sedative properties and addition of enrichment material to the pens; (2) to evaluate consumers' sensory acceptability of meat from these different strategies; and (3) to determine the effect of three factors (feeding supplementation, production system and price) on consumer's purchasing intention of pork meat.

2. MATERIALS AND METHODS

2.1. Animal and housing conditions

Fifty-six male pigs [(Landrace x Large white) x Pietrain] were used in the present experiment. Pigs arrived at the experimental farm with an average live weight of 25 ± 0.27 kg (mean ± SE) and ten weeks of age and were kept together until the experiment started at the age of 16 weeks (and average weight of 49.8 ± 0.56 kg). Pigs were randomly divided in four different treatments (14 pigs/treatment) consisting in a) pigs supplemented with both enrichment material and herbal compound (EEHC), b) pigs supplemented with enrichment material (EE), c) pigs supplemented with the herbal compound (HC) and d) control group (CG). Two replicates of 7 pigs per treatment were carried out in two different modules with all treatments in each module.

Pens were equal in size (13.67 m^2), and were divided by means of metallic fences, allowing visual contact to the other pens. The space availability per pig was 1.95 m^2 and food and water were provided *ad libitum* by means of one drinker and two hopers. The enrichment material used in EE and EEHC consisted in natural hemp ropes, sawdust and rubber balls, all provided at the same time during all the experiment. Two hemp ropes of 80 cm were hanged in the walls of environmentally enriched pens and replaced when the length was lower than 30 cm. Furthermore, 1/3 of the slats of

EE and EEHC pens were covered with polypropylene sticks (Click-in®, Rotenca, Agramunt, Spain) in order to support the sawdust, and new sawdust was added every two days. A single rubber ball with a diameter of 15 cm was also provided in each EE at the beginning of the experiment. The herbal compound used (Sedafit ESC, Phytosynthèse, Saint-Bonnet de Rochefort, France) contained *Valeriana officinalis* and *Passiflora incarnata*, both with sedative properties, and it was manually added to the food concentrate of the HC and EEHC treatments in a concentration of 2000 mg/kg. Behaviour observations, body weights and skin lesions were periodically registered, and blood, saliva and hair samples were collected as part of a broader study.

2.2. Carcass quality measurements

All the animals were slaughtered in a commercial abattoir at the age of 24 weeks with a mean live weight of 109.77 ± 1.35 kg. All pigs were gas stunned before sticking. Carcass weight was measured 45 minutes *post mortem* (p.m.), and carcass yield was calculated dividing carcass weight and live body weight. Furthermore, backfat (LR3/4FOM) and muscle thicknesses (MFOM) were measured between the 3rd and 4th last ribs, at 6 cm from the midline, using a Fat-O-Meat'er probe (Carometec A/S, Herlev, Denmark). These values were used to calculate the lean percentage according to the Spanish official equation (Font-i-Furnols & Gispert, 2009). Twenty-four hours p.m., minimum fat and skin thickness (perpendicular to the skin) were measured over the *gluteus medius* muscle (MLOIN) using a ruler on the left carcass of each animal. Loin and carcass length were also measured, using a tape. Loin was measured from the atlas to the first lumbar vertebra, while carcass was measured from the first rib to the anterior edge of the pubic symphysis.

2.3. Meat quality measurements

At 24 hours p.m., muscle pH and electrical conductivity were measured in the *Longissimus thoracis* (LT) at the last rib level using a Crison portable meter (Crison, Barcelona, Spain) equipped with a Xerolyte electrode, and a Pork Quality Meater (PQM-I, INTEK Aichach, Germany), respectively. At the same moment, colour of the exposed cut surface of the LT muscle at the last rib level was evaluated by three trained observers using the Japanese scale colour (1: very pale to 6: very dark) (Nakai *et al.*, 1975) The colour parameters on the CIELab space (CIE, 1976): luminosity L*, tendency to red a* and tendency to yellow b* were also obtained by means of a Minolta Chromometer (CR-400, Minolta Inc., Osaka, Japan). Drip loss was also evaluated from the LT according to the methodology described

by Rasmussen and Andersson (1996). Marbling was evaluated by three technicians using the National Pork Production Council pattern (NPPC, 1999) on the LT muscle at the last rib level. Furthermore, LT samples were also taken for further analysis of intramuscular fat and texture and frozen at -20ºC. Intramuscular fat content (GRINLD) was analysed with infrared FoodScan equipment (FOSS analytical, Hillerød, Denmark) at wavelengths between 850 nm and 1050 nm. Samples were thawed for 24 h at 2ºC, and grinded using a Robot-Coupe Blixer 3 blender (Seysant Atlantic S.L., Soria, Spain). Shear force was determined in LT samples from the 2nd–3rd last rib level. LT was thawed during 24 h at 2ºC. Then, chops were cooked in a pre-heated oven to 110ºC (Spider 5, Novosir, Spain) until the internal temperature reached 75º C. After 2 h at room temperature, pieces of 3 x 1.5 x 1.5 cm were collected for the analysis. Then, pieces were sheared using an Alliance RT/5 texture analyser (MTS Systems Corp., Eden Prairie, MN, USA) equipped with a Warner-Bratzler blade with crosshead speed set at 2 mm/s, and peak load (kg) was recorded.

2.4. Consumers' study

A total of 110 consumers participated in the study which was performed in Barcelona. Consumers were selected to be representative of the Spanish population according to gender and age although a slight bias in gender was finally obtained (Table 1). A total of 11 sessions of ten consumers each were conducted.

First, a sensory analysis acceptability test was performed to determine if consumers were able to find differences in acceptability depending on the types of loin. Loin sections of 1.5 cm thick were cooked in a pre-heated oven at 200ºC until the internal temperature reached 76ºC, which is the temperature recommended for sensory evaluations (Bejerholm & Aaslyng, 2003). Once cooked, each slice was divided in four pieces of 1.5 cm-thick (perpendicular to the subcutaneous fat), and were covered with aluminium foil, codified with a three-digit code, and kept warm until being served to the consumers. Each consumer received four samples, one from each treatment, that were evaluated in blind conditions. Samples were distributed to the consumers monadically and following a design to avoid the first sample and carry over effect (MacFie *et al.*, 1989). Consumers evaluated the overall acceptability/liking, tenderness, smell liking and flavour liking according to a nine-point scale (from 1: 'dislike very much'/ 'very hard', to 9: 'like very much' / 'very tender') without the intermediate level (5: 'neither like nor dislike').

Table 1: Socio-demographic characteristics of the consumers (n=106)1

	Overall	Cluster 1	Cluster 2	Cluster 3
Consumers (%)	100	26.41	51.89	21.70
Gender (%)				
Women	62.3	39.3[a]	69.1	73.9
Men	37.7	60.7[b]	30.9	26.1
Age (%)				
<26	5.7	7.1	3.6	9.1
26-40	30.5	39.3	29.1	22.7
41-55	28.6	35.7	25.5	27.3
56-70	32.4	17.9	40	31.8
>70	2.9	0	1.8	9.1
Finished levels of studies (%)				
Primary school	8.7	3.6	11.3	8.7
Secondary school	55.8	50	50.9	73.9
University	35.6	46.4	37.7	17.4

[1] Four consumers out of the initial 110 were not considered because information was not provided.
[2] Different letters within cluster and demographic characteristics indicate significant differences between the overall distribution and the distribution within the cluster.

Second, a preference study was carried out by means of a conjoint analysis. Three factors were analysed: feeding, production system, and price. Thus, for feeding, two levels were introduced: a) conventional feed, and b) conventional feed supplemented with natural herbs with a relaxant effect. For production system, there were also two levels: a) conventional farming system and b) conventional farming system with improvements for the animal welfare. Finally, three levels were considered for price: 3€/kg, 5€/kg and 7€/kg. A full design was used, thus 12 different profiles were obtained from all the possible different combinations of the levels of each factor. Consumers were asked to look at the cards carefully and rank them according to their purchase intention from 1 (less preferred) to 12 (most preferred). Four consumers were not considered in the conjoint analysis because information was not properly provided. Consequently, the analysis was performed using 106 subjects.

2.5. Statistical analysis

Statistical analyses were conducted using Statistical Analysis Software (SAS version 9.2; SAS institute Inc., Cary, NC; USA). Significance was established at $p<0.05$ for all the analyses, while a tendency was considered between $p>0.05$ and $p<0.1$.

For carcass and meat quality parameters, the dependent variables were analysed by means of general linear models (MIXED procedure). Use or not of herbal compound, environmental enrichment and their interaction were considered as fixed effects. For body weight, sample day was included as a fixed effect, and interactions of the previous factors with sample day were added to the model. Carcass weight was included in the model as a covariate in carcass quality traits if significant. The least square means of fixed effects (LSMEANS) with Tukey adjustment were used for comparisons when analysis of variance indicated differences ($p<0.05$).

For the consumer's conjoint analysis, a Ward method was used in an agglomerative hierarchical cluster analysis (PROC CLUSTER) with the intention to determine different segments of consumers according to the ratings given to the cards. Clusters were selected from the dendogram obtained, trying to maintain as much as homogeneity within each cluster but at the same time, maximizing the heterogeneity between clusters. Conjoint analysis was analysed for all the consumers as a pool and by cluster using the TRANSREG procedure. Demographic differences among different clusters were analysed using the GraphPad QuickCalcs website for statistics by means of the chi-square test two by two (http://graphpad.com/quickcalcs/chisquared1/).

Sensory attributes of meat scored by consumers were analysed using the MIXED procedure by cluster and for all the consumers as a pool. The model included use or not of environmental enrichment and herbal compound and its interaction as fixed effects, consumer was included as a random effect and session as blocking variable. Lsmeans adjusted to Tukey were used to analyse the differences of the interactions.

3. RESULTS

3.1. Meat and carcass quality

Body weight previous to slaughter was significantly lower (p=0.049) in the CG (105.11± 1.17 kg) compared with EE (111.79 ± 2.49 kg; p=0.0005), HC (110.04 ± 3.04 kg; p=0.0115) and EEPH (112.35 ± 2.39 kg; p=0.0004). Furthermore, yellowness (b*) was higher in the pigs with environmental enrichment (p=0.004). No other significant differences were observed in terms of carcass and meat quality (Table 2), although a tendency was observed in the minimum fat thickness over the *gluteus medius* muscle (p=0.060), with a mean value of 10.00±0.37 mm for pigs supplemented with herbal compound and 11.07±0.6 mm for pigs with conventional feed.

3.2. Consumers' study

As previously described, consumers scored the sensory characteristics of the meat. No significant differences were observed in any of the different characteristics assessed for neither pigs supplemented with the herbal compound nor the animals supplied with environmental enrichment. Averaged values for overall acceptability, tenderness, odour and taste were 5.60 ± 0.08, 4.90 ± 0.09, 5.91 ± 0.07 and 5.75 ± 0.08, respectively. When clusters were studied individually, similar results were obtained and no significant differences between treatments were obtained.

Table 2: Least square means of meat and carcass quality traits from pigs reared or not with environmental enrichment and supplemented or not with an herbal compound with sedative properties.

	Housing system[b]		Herbal compound[c]			P-values[d]	
	BE	EE	no	yes	SEM[a]	HS[b]	HC[c]
Carcass quality traits							
Live weight (kg)	107.57	112.07	108.45	111.19	1.35	0.0002	0.02
Carcass weight (kg)	80.88	82.39	80.89	82.38	1.02	0.37	0.38
Carcass yield (%)	75.18	73.78	74.57	74.38	0.20	0.07	0.58
Last rib backfat (mm)	15.17	15.26	15.30	15.12	0.35	0.86	0.72
Muscle thicknesses (mm)	57.18	58.74	57.68	58.23	0.60	0.14	0.60
Carcass lean meat (%)	61.58	61.79	61.55	61.82	0.30	0.70	0.62
Carcass length (cm)	83.07	82.80	82.90	82.97	0.37	0.56	0.87
Loin length (cm)	84.33	84.15	84.32	84.16	0.37	0.74	0.76
MLOIN	10.30	10.72	11.10	9.91	0.35	0.51	0.06
Conformation	2.72	2.64	2.63	2.73	0.06	0.54	0.50
Meat quality traits							
pHuLT	5.59	5.58	5.59	5.58	0.01	0.70	0.64
ECuLT	4.33	4.40	4.39	4.34	0.08	0.69	0.82
Lightness L*	48.28	49.17	48.52	48.93	0.25	0.11	0.44
Redness a*	6.93	7.00	6.86	7.07	0.11	0.75	0.36
Yellowness b*	1.25	1.72	1.36	1.60	0.10	0.04	0.28
Colour EJC	2.44	2.24	2.35	2.33	0.06	0.13	0.85
Drip loss	5.90	5.90	5.87	5.92	0.22	0.99	0.91
Marbling NPPC	1.52	1.46	1.50	1.48	0.06	0.65	0.86
Intramuscular fat %	2.11	2.06	2.09	2.07	0.04	0.58	0.84
Shear force (g/cm^2)	5.30	5.65	5.42	5.30	0.12	0.58	0.77

MLOIN: minimum fat over the *gluteus medius*. pHuLT: muscle pH at *Longissimus thoracis* 24 h p.m.; ECuLT=electrical conductivity measured in the *Longissimus thoracis*, EJC: subjective colour using a Japanese colour scale ; Marbling NPPC: subjective marbling with pattern from National Pork Producers Council (means of 3 assessors); L*, a*, b*: objective measure of the colour with the Minolta Chromameter

[a] SME: Standard error of the mean

[b] Housing system (HS) BE=Barren environment/ EE= enriched environment.

[c] Herbal compound (HC) yes=supplied with herbal compound/ no= not supplied.

[d] P-values: ET: environmental enrichment significance, HC: herbal compound significance. Interaction HS*HC was not significant for any parameter.

Table 3 shows the results of the conjoint analyses. When all the consumers were studied as a pool, the most important factor (45.7% of importance) was the production system, with a preference for those systems with improvements for the animal welfare as indicated by the higher utility value. The second factor considered by the consumers (34.4% of importance) was the feeding system, with preference for conventional feed supplemented with natural herbs with relaxant properties. The less important factor was the price, being the intermediate price the most preferred.

Table 3: Relative importance and utility values for the total of the consumers and for different clusters.

	Overall	**Cluster 1**	**Cluster 2**	**Cluster 3**
-Feeding supplementation				
Conventional food	-1.0	-0.7	-2.0	1.6
Supplemented food with natural herbs	1.0	0.7	2.0	-1.6
Relative importance (%)	**34.4**	**14.3**	**45.9**	**38.4**
-Production system				
Conventional farming system	-1.3	-0.7	-1.7	-0.5
Conventional with animal welfare improvements	1.3	0.7	1.7	0.5
Relative importance (%)	**45.7**	**13.7**	**40.4**	**13.2**
-Price				
3 €	0.2	3.3	-0.5	-1.9
5 €	0.5	0.6	0.7	0.0
7 €	-0.6	-4.0	-0.2	2.0
Relative importance (%)	**19.9**	**72.0**	**13.7**	**48.4**
RMSE	3.0	1.3	2.2	2.6
R^2	0.2	0.8	0.6	0.4

RMSE: Root mean square error
R^2: Coefficient of determination

Consumers from this study were clearly divided in three different clusters (Table 3). Cluster one was composed by 26.4% of the consumers, and the most important factor for them was the price (72% of importance), being the most preferred the lower price. The other attributes were poorly considered showing a 14.3% of importance in feed supplementation, and a 13.7% of importance in

the production system. This cluster presented a higher proportion of men (p=0.01) compared with the total distribution (Table 1).

Cluster 2 was the biggest, with half of the consumers (51.8%). Feeding (45.6% of importance), and production system (40.4% of importance) were the most important factors for the consumers of this cluster, with preference for food supplemented with herbs, and for animal welfare improvements, respectively. Price was the least important attribute for this group (13.7%), and showed a preference for the intermediate price. Cluster 2 followed a similar gender, age, and level of studies distribution compared with all the consumers' evaluated.

Finally, cluster 3 (21.7% of consumers) considered price as the most important factor (48.4% of importance), with preference for the highest price, although feeding system was also important for this cluster (38.4% of importance), with preference for conventional feeding. Production system was considered the least important attribute for this group, although they preferred those systems reared with enrichment material. Consumers from this cluster tended to have less university and more secondary studies than the overall population (p=0.07).

4. DISCUSSION

The results presented are part of a broader study aiming (1) to evaluate the effects of the mitigation of chronic stress by means of environmental enrichment and herbal compounds on behaviour and physiological indicators, and (2) to assess the effect of those strategies on meat and carcass quality, and consumer's preference and acceptability in growing pigs and pork meat. The second aim is discussed below.

4.1. Carcass and meat quality

Limited effects were found in the carcass and meat quality traits in relation with enrichment material and the herbal compound. The only difference reported in carcass traits was an increased body weight before slaughtering in EE, HC and EEHC compared with CG. These differences may suggest a better growth when environmental enrichment and/or herbal compounds are provided. Although previous studies with environmental enrichment have reported conflictive results, in general, the provision of straw improves performance (e.g. Beattie *et al.*, 2000; Lyons *et al.*, 1995). However, when point source objects are provided, these differences are less consistent (Van de Weerd and

Day, 2009). Potential explanations for the increase in performance when providing enrichment material could be related to two theories. First, Lyons *et al.* (1995) argued that by increasing exploratory behaviour, enrichment material could enhance interactions with feeders and, in turn, food consumption. Secondly, the reduction of stress associated with the possibility to perform highly driven behaviours such as foraging (Schaefer *et al.*, 1990), could be related to lower levels of catabolic hormones such as catecholamines and cortisol (Mormède *et al.*, 2007). Along that lines, the levels of cortisol measured in the pigs of the present experiment were found to be lower for the enriched pigs (Casal *et al.*, 2014). Up to the authors' knowledge, no studies have reported the effect of supplementation with herbal compounds on body weight.

Yellowness was higher in the animals reared in enriched environments, although, the differences were not relevant because probably they would not be detected by human eye using the subjective colour evaluation. These results show that there is no clear influence of treatment on this particular parameter.

In agreement with previous studies evaluating the effects of environmental enrichment, no other parameters analysed of carcass and meat quality traits presented significant differences (Geverink *et al.*, 1999; Hill *et al.*, 1998). Nevertheless, contrarily to the present results, a better water-binding capacity has been reported in pigs reared in enriched environments (Beattie *et al.*, 2000; Klont *et al.*, 2001; Støier *et al.*, 2001).

4.2. Consumer's intention to purchase

Conjoint analyses are used to obtain information about how the attributes of the product analysed can affect liking and/or purchase intention (Næs *et al.*, 2001). It consists in combining the different attributes, and allowing consumers to sort the different profiles by preference. The objective of conjoint analysis is to determine the relative importance of each attribute from the combinations of attributes scored by consumers (García-Torres *et al.*, 2016). Usually the behaviour of the consumers in front of a conjoint analysis allows identifying different segments or clusters of consumer.

In the present study, the most determinant attribute when all the consumers were considered together was the production system, followed by feeding supplemented with herbs and the least important attribute was the price. Previous studies also reported a preference and an increased willingness for animal friendly products (Napolitano & Girolami, 2010). However, although a 77% of European consumers expressed a concern in animal welfare (Kanis *et al.*, 2003), consumers seem to

be more influenced for other factors such as origin, flavour, price and meat quality (McEachern *et al.*, 2007).

Cluster one was mainly composed by men whilst the majority of consumers from clusters two and three were women. Our results are in line with previous studies, reporting a higher influence of meat price in men, with preference for lower prices. (e.g. Font-i-Furnols *et al.*, 2011), whilst women presented a higher concern in animal welfare as previously reported, probably associated with a higher implication in household tasks related with animals (Vanhonacker *et al.*, 2007).

Cluster two was the segment with the highest interest for animal welfare improvements. This cluster presented a preference for intermediate prices probably associated with the general perception that cheap products are considered to come from unreliable origins (García-Torres *et al.*, 2016), which is clearly related with an increased willingness to pay for improved animal welfare products (Harper and Henson, 2001). Price was considered as the most important attribute for both cluster 1 and cluster 3, but whilst in cluster 1 there was a preference for the cheapest meat, cluster 3, preferred the highest price. Similar results were reported in beef meat by Sasaki et al (2006), where some clusters showed a preference for the lowest price, but other preferred the highest price. However, other studies such as Font-i-Furnols *et al.* (2011) in lamb meat and Mesías *et al.* (2005) in beef meat, showed a preference for the lower prices in all the segments.

Surprisingly, cluster 3 presented a preference for a conventional production system and for the most expensive meat. The general perception that higher prices are related to a better product may explain the interest for more expensive prices, suggesting that this cluster might be more interested in quality aspects than in animal welfare improvements. Another possible hypothesis to explain the existence of this group is somehow related with the cluster described by Dutra *et al.* (2013), who distinguished a group of consumers in a study from China which were not interested in the way pigs are raised, they called this cluster the *indifferent,* and they related this group with urbanites that not only know little about pig production, but also are not interested in acquiring this knowledge.

The results presented here have a few limitations. First of all, the number of subjects in the consumers´ study should be higher in order to draw clear conclusions, and the gender ratio may be more balanced. Furthermore, consumers mainly came from urbanized areas, with less knowledge in pig production systems and with an increased concern for animal welfare than in rural areas (Kendall *et al.*, 2006). Overall, the present results show the importance of animal welfare for consumers. However, a broader study taking into consideration the limitations described before would be required to draw more general and definitive conclusions.

5. CONCLUSIONS

Animal welfare is an important attribute considered by pork meat consumers, with a preference for systems aiming to increase the welfare. Women appear to be more concerned about animal welfare, with few considerations in price, while for men, price is also a very important factor to consider. Neither environmental enrichment nor herbal compound affected the quality of the carcass and the meat, although pigs supplied with herbal compound, environmental enrichment or both, presented a higher weight compared with control group. Nevertheless, a more balanced study with more subjects and more attributes considered should be carried out before drawing definitive conclusions.

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General Discussion

GENERAL DISCUSSION

Stress is a multi-factorial process which can be induced by a high diversity of different stressors (Wiepkema and Koolhaas, 1993), with different effects depending on their magnitude and chronicity (Chrousos, 2009). Since not all stress responses produce the same changes, different stress indicators are not appropriate in all the situations (Broom, 2011). Thus, the response produced in front of an acute stressor will be very different than the response produced in chronic stress exposure and, consequently, the parameters which better reflect those different responses may vary. Moreover, the perception and response to such stressors are variable, producing high individual differences in front of the same stimuli (McEwen and Stellar, 1993). To add further complication, some changes are not only related with stress, and neutral or even pleasant situations may produce stress-like changes (Mason and Mendl, 1993). Overall, because of the non-specificity of this biological response, which includes multiple behavioural and physiological changes (Chrousos, 2009), it can hardly be analysed by means of a single measure.

The main objective of this thesis was to identify non-invasive physiological indicators of chronic stress exposure in growing pigs. With this aim, two main experiments were performed. The first one aimed to evaluate the feasibility and reliability of hair as a matrix to detect cortisol which has previously been described as a good chronic stress indicator in other species (Accorsi *et al.*, 2008; Davenport *et al.*, 2006; Kirschbaum *et al.*, 2009). Results from this experiment are described in chapter 1. The second experiment evaluates the effects of two strategies aimed at stress reduction (provision of environmental enrichment and herbal compounds) over physiological indicators of stress (chapter 2), behaviour and performance (chapter 3), and finally, carcass and meat quality traits, and consumers' perception (chapter 4). Thus, chapter 1 and chapter 2 analysed the feasibility and reliability of different physiological indicators of chronic stress, and, additionally, these physiological indicators analysed in chapter 2 were contrasted with behaviour and performance parameters in chapter 3 and with meat quality indicators in chapter 4. Furthermore, consumers' preference and acceptability were assessed to evaluate the possible societal impact of improving animal welfare by means of stress reducing strategies.

1. PHYSIOLOGICAL INDICATORS OF CHRONIC STRESS EXPOSURE

The interest for non-invasive indicators of stress has increased considerably during the last years. The hypothalamic pituitary adrenocortical (HPA) axis and the sympathetic adrenomedullary (SAM)

system are generally considered to be the two key players in the stress response (Koolhaas *et al.*, 2011). Thus, the physiological response produced as a consequence of the activation of both systems can be used objectively as an indicator of stress exposure. Acute stress may be measured by means of different biomarkers related with the activation of the SAM system such as the same catecholamines but also other proteins like IgA, CgA and α-amylase (Obayashi, 2013). Furthermore, blood cortisol and saliva increases a few minutes after stress exposure as a consequence of the HPA axis activation and may be used also as indicators of acute stress (Mormède *et al.*, 2007). However, there is not an accepted "golden standard" biomarker for chronic stress assessment (Russell *et al.*, 2012). Nevertheless, cortisol seems to be the best candidate. Previous studies tried to evaluate chronic stress by means of multiple samples in different matrix such as blood, saliva, urine and faeces. However, none of these approaches provides a truly long-term index of HPA axis activity (Meyer and Novak, 2012). During the last years, hair has drawn scientific attention as a promising approach for the retrospective assessment of long-term cortisol exposure (Manenschijn *et al.*, 2011). Furthermore, it is a non-invasive and a painless method, sampling may be performed by a non-professional, and may be stored at room temperature before the analysis (Gow *et al.*, 2010). Although different studies have been carried out in different species, up to date, hair has only been assessed twice in pigs (Bacci *et al.*, 2014; Martelli *et al.*, 2014), both times during the development of this thesis.

1.1 Hair cortisol

Although hair cortisol had been tested and linked to long-term HPA activity in different species (Stalder and Kirschbaum, 2012), no data was available for pigs when the present investigation was started. Therefore, a validation like the one carried out in chapter 1 was required (Buchanan and Goldsmith, 2004). Furthermore, in the second experiment performed, a different kit was used and the analytical procedure was slightly different, which required another validation (chapter 2). The technical validation performed in both chapter 1 and chapter 2 demonstrated the feasibility of cortisol detection in the hair of growing pigs, with similar specificity, linearity, accuracy and precision than those previously reported in other species (e.g. Bennett and Hayssen, 2010; Davenport *et al.*, 2006; Manenschijn *et al.*, 2011; Yamanashi *et al.*, 2013).

Previous studies reported different hair cortisol levels between different body regions (Ashley *et al.*, 2011; Carlitz *et al.*, 2015; Li *et al.*, 2012; Macbeth *et al.*, 2010; Terwissen *et al.*, 2013; Yamanashi *et al.*, 2013). In chapter 1, these differences in pigs were also confirmed, finding significant higher levels

in the loin part of the dorso–lumbar (L) region compared with the dorsal area of the neck (D). These results suggest that the region should be taken into account, especially when repeated samples are assessed. Although small amounts of hair are required to analyse cortisol, in pigs it may be difficult to obtain the intended amount of sample. Consequently, in the second experiment (chapter 2) all samples were obtained from L region, because less samples had to be discarded from this region in chapter 1, and pigs usually present a higher amount of hair in L region compared to D region.

Cortisol levels reported in chapter 1 were slightly higher than those reported in chapter 2 at a similar age (17 and 16 weeks of age, respectively). Furthermore, the concentration of cortisol increased moderately in chapter 1 in the second sample obtained, when pigs were 21 weeks of age, whilst decreased at the second experiment at both 20 and 24 weeks of age. A possible hypothesis to explain the differences reported in chapter 1 and chapter 2 is related with the stress response produced by the different experiments. In chapter 1, animals were weekly regrouped, which is supposed to produce stress to the animals (Ekkel *et al.*, 1997). Thus, it is fair to assume a certain induced stress in those pigs, with the consequent increase in the HPA axis activity. On the other hand, in chapter 2, the objective of the different treatments was to reduce the stress of the pigs, which may explain the decreased levels of hair cortisol observed. Age has also been related to cortisol levels, presenting normally older individuals lower levels in plasma and saliva (De Jong *et al.*, 2000; Kirkwood *et al.*, 1987; Ruis *et al.*, 1997). Although no control group was available in chapter 1 to disentangle the age effect, the increment of hair cortisol could be triggered by the remixing procedure. Furthermore, the decrease of hair cortisol reported in chapter 2 when implementing strategies to reduce stress suggest that hair cortisol concentration may provide information on HPA activity in front of the divergent stress stimuli applied.

On the other hand, the higher levels of cortisol observed in control group compared with treatments aiming to stress reduction (chapter 2), were only reported at 24 weeks but not at 20 weeks of age. We think that it should be explained by the shaving procedure. In the second experiment, hair was shaved four weeks before each sample (as we recommended in chapter 1) to ensure a similar length and age of the hair collected between two consecutive samples. However, part of the hair analysed did not belong to the inter-samples periods, since we did not consider the hair under the skin. It has been described that the deep of the hair in growing pigs is about 3-4 mm (Mowafy and Cassens, 1976). Assuming that cortisol can be incorporated into the growing hair cells via passive diffusion (Stalder and Kirschbaum, 2012), 3-4 mm of hair remained in the skin after cutting and were analysed in the next period. Thus, the sample taken at the age of 20 weeks, was composed by 3-4 mm of hair containing cortisol information from the period before starting the experiment (< week 16 of age)

covering up the effect of the treatments. According to Bacci *et al.* (2014), the proportion of hair remaining in the skin belongs to 15 days approximately. However, our hypothesis for the present study is that this time was probably about 7 to 10 days, since hair growth was between 10 and 14 mm per month (data not published, based in personal observations).

1.2. Chromogranin A

Measurement of CgA in saliva has been used as an indicator of SAM system activity (and, hence, of acute stress), as an alternative to catecholamines adrenaline and noradrenaline (Martínez-Miró *et al.*, 2016). In pigs, CgA has been analysed in different situations such as immobilization with a nose snare (Escribano *et al.*, 2013), food deprivation (Ott *et al.*, 2014) and isolation and regrouping (Escribano *et al.*, 2015). However, before this work, it had never been analysed as a chronic stress indicator, up to the authors' knowledge. Results from chapter 2 shown no differences at the beginning of the experiment when the animals were aged 16 weeks, but differences were reported at the age of 20 and 24 weeks of age, when pigs were subjected to the different treatments, with lower levels shown by pigs reared with different strategies aimed to reduce stress. Our results suggest that CgA may be a useful biomarker of chronic stress in growing pigs, although more studies would be required (using other chronic stressful stimuli) to confirm this hypothesis.

CgA has been suggested to be stored in the dense core granules of the adrenal medulla and of many neuroendocrine cells and neurons (O'Connor and Frigon, 1984), but also in secretory granules in acinar cells (Kanno *et al.*, 2000). Our hypothesis to explain the reported differences is related with the abovementioned accumulation of CgA, which may be higher in situations of chronic stress, since more CgA may be produced and stored in these vesicles affecting also the baseline levels of CgA in the animals.

Furthermore, we suggest that the vesicles containing CgA could present a similar hyper-reactivity than those suggested for the adrenal cortex in chronic stress situations (Rushen, 1991). However, more research is needed to understand the mechanisms involved in the production and secretion of CgA to better understand the relation with chronic stress.

1.3 Salivary cortisol and TNF-*α*

In chapter 2, cortisol was also assessed in saliva as a biomarker of HPA response in order to compare with hair values. Furthermore, immune function has also been said to be impaired after exposure to stress (Glaser *et al.*, 1987), and for this reason, it was considered relevant to obtain an indicator of the immune function. However, it was not possible to analyse the cellular immune response, and although the main interest of this thesis was focused in non-invasive biomarkers, blood samples were collected to quantify the levels of TNF-α. Contrarily to our hypothesis, no differences were reported (chapter 2) in neither salivary cortisol nor TNF-α in the control animals compared with those subjected to the treatments aimed to reduce stress.

Salivary cortisol has been previously related with a blunted circadian rhythm (De Jong *et al.*, 2000) and with a higher baseline concentration (De Jong *et al.*, 1998) in pigs subjected to chronic stress. A potential explanation for the lack of effect in the present experiment may be that the intensity of the tretments aiming to reduce the stress were not sufficient to be reflected in a decrease of baseline levels of salivary cortisol. Although more studies should be carried out, our results reinforce the findings that salivary cortisol is a proper acute stress indicator, but not so sensitive probably towards chronic stressors.

On the other hand, inconsistent results have been previously reported in relation with TNF-α and chronic stress. As an example, two different studies reported completely different results in students with high levels of anxiety (Chandrashekara *et al.*, 2007; Maes *et al.*, 1998). The present results suggest that other immune function indicators, like ratio lymphocites/neutrophyles, would probably be more appropriate to establish comparisons between HPA activity and immune system in chronic stress situations.

1.4 Relation between biomarkers

Hair cortisol presented a moderate correlation with CgA (r=0.48, chapter 2) adding support to our theory that both biomarkers may be used as indicators of chronic stress. Furthermore, the other expected indicator related with chronic stress, TNF-α, presented a low (but significant) correlation with both hair cortisol (r=0.33) and salivary CgA (r=0.36). However, with the present results, we cannot explain why no differences were reported in TNF-α levels between the different treatments (chapter 2).

A low correlation was also reported between CgA and salivary cortisol (r=0.27), this value is lower than those reported by Escribano *et al.* (2013) et by Ott *et al.* (2014) and higher than the lack of correlation described by Escribano *et al.* (2015) in growing pigs. We suggest that correlations may be stronger when an acute stress response is triggered. Along that line, both Escribano *et al.* (2013) and Ott *et al.* (2014) subjected pigs to a punctual stressor in order to produce an acute stress response, while in the present study no stress was produced to the animals. Thus, while we compared the basal levels of both biomarkers, the other studies measured an acute stress response.

As found in other studies (e.g. Sauvé *et al.,* 2007), no correlations between salivary and hair cortisol were reported in neither chapter 1 nor chapter 2, supporting the idea that both biomarkers are appropriate to measure different stress responses (acute vs. chronic, respectively). Previous studies reporting correlations between both matrix have taken multiple samples of saliva (D'Anna-Hernandez *et al.*, 2011; van Holland *et al.*, 2012), measuring more precisely the basal levels of salivary cortisol.

2. MEASURES AIMING TO REDUCE THE STRESS

The main objective of the second experiment (chapters 2, 3 and 4) was to measure chronic stress by improving the environmental and management conditions of the pigs. Other studies aiming to assess stress response have induced stress providing negative stimulus to the animals (e.g. Kanitz *et al.*, 2005; Merlot *et al.*, 2011; Tuchscherer *et al.*, 2010). However, as it has been widely investigated intensive farm production systems may hamper the welfare of the animals and induce stress per se. Thus, it was decided to evaluate chronic stress using strategies previously suggested to improve welfare rather than creating a chronically stressful scenario, less acceptable from an ethical point of view. We acknowledge that inducing stress may be more adequate to study acute stress response, and would be also interesting in order to validate the biomarkers studied, especially CgA.

2.1 Environmental enrichment

Previous experiments comparing different housing conditions had focused basically in behavioural changes, reporting more signs of chronic stress in those pigs subjected to barren environmental conditions compared with pigs housed under environmental enriched conditions (for a review see: Van de Weerd and Day, 2009). However little attention has been paid to physiological indicators in relation with the rearing environments. In the present thesis (chapter 2, 3 and 4), pigs were reared

in either an enriched environment consisting of sawdust, natural hemp ropes and rubber balls (all materials provided at the same time) or in a barren environment.

The effects of barren housing conditions on pig behaviour have been well discussed in previous studies. It was shown that barren conditions hamper the development of appropriate behaviour, while they may increase abnormal behaviours (Young, 2003). In chapter 3, we found that pigs reared in enriched conditions presented more exploratory behaviour compared with pigs reared in barren conditions as it was previously reported (Beattie *et al.*, 1995; Melotti *et al.*, 2011; Pearce and Paterson, 1993), suggesting that these pigs were more stimulated (Van De Weerd *et al.*, 2003). Moreover, as it has been said before, the frustration of normal exploratory behaviour by barren environments can lead to abnormal behaviours such as stereotypies and redirected behaviours in an attempt to satisfy their exploratory needs (Pearce and Paterson, 1993; Studnitz *et al.*, 2007). In the present study, more stereotypies and redirected behaviour were reported confirming this theory. Furthermore, pigs reared in enriched conditions spent more time active compared with pigs reared in barren conditions as it was previously reported (Beattie *et al.*, 1995; Bolhuis *et al.*, 2005; Morgan *et al.*, 1998).

As it was predicted, the levels of hair cortisol and CgA were lower in the animals with environmental enrichment (chapter 2) which are supposed to experience less stress than animals reared in barren conditions. Our results could not be compared since no other studies were found linking hair cortisol or CgA with environmental enrichment. However, previous studies have reported discrepancies on the effect of providing environmental enrichment on salivary cortisol levels. Whereas de Jong *et al.* (1996) and Merlot *et al.* (2012) reported lower levels of salivary cortisol on enriched pigs, de Jong *et al.* (1998) and de Groot *et al.* (2000) found higher levels, and Morrison *et al.* (2007) did not reported differences between housing environments. These discrepancies may be again related to the probably higher sensitivity of salivary cortisol as acute stress indicator. Exploratory behaviour may be related to activity and arousal and, thus, it may be possible that depending on the sampling procedure, salivary cortisol levels could be reflecting either the positive effects of enrichment on allowing motivated behaviours to be performed, or the activity itself produced by exploration.

Pigs reared in environmental enriched conditions presented higher body weight at the end of the experiment (chapter 3 and chapter 4) than pigs reared in barren conditions, as it has been reported previously (Beattie *et al.*, 2000; Lyons *et al.*, 1995; Schaefer *et al.*, 1990). This finding may be explained by the lower levels of cortisol reported in the animals reared with environmental enrichment (chapter 2): Lower levels of corticosteroids may result in improved growth performance

(Schaefer *et al.*, 1990) because of a reduction in the catabolic activity of cortisol (Mormède *et al.*, 2007). Moreover, more contacts with the feeder with the subsequent greater consumptions may be expected as a consequence of the higher activity and exploratory behaviour (Lyons *et al.*, 1995) reported in the present experiment, which may also contribute to increase the body weight of the animals.

No general differences were observed in carcass and meat quality traits (chapter 4) as it has been previously reported (Geverink *et al.*, 1999; Hill *et al.*, 1998). However, other studies found a better water-binding capacity in pigs reared in enriched conditions (Beattie *et al.*, 2000; Klont *et al.*, 2001), which may be explained for the depletion of glycogen detected in chronic stress situations, since glycogen in muscle is capable of binding water (Fernandez and Tornberg, 1991). Moreover, it has been suggested that animals reared in enriched environments may cope better with new stressful stimuli like those found during the ante-mortem period. It is possible that the beneficial effects of environmental enrichment on meat quality may be more pronounced when animals are subjected to more stressful pre-slaughter conditions than the ones experienced in the present work. Furthermore, no differences were detected in the acceptability, tenderness, odour and taste of the meat reared with environmental enrichment.

2.2 Herbal compounds

A herbal compound containing Valerian (*Valeriana officinalis*) and Maypop (*Passiflora incarnata*) was tested also in chapters 2, 3 and 4 with the aim to reduce the stress of the pigs, since both plants have different bioactive compounds with sedative and/or relaxing properties (Dhawan *et al.*, 2001; Murphy *et al.*, 2014). Up to the knowledge of the authors, the effect of herbal compounds on pigs' welfare has only been published twice (Peeters *et al.*, 2006, 2004). In both studies, the herbal compound used was the same than the one used in the present work (Sedafit ESC, Phytosynthèse, Saint-Bonnet de Rochefort, France).

A reduced number of skin lesions was reported in the animals supplemented with the herbal compound (chapter 3). Our results are in disagreement with those found by Peeters *et al.* (2006), who reported more lesions in those pigs supplemented with Sedafit (contrarily to their prevision). Whether these divergences may be explained by differences in the experimental design or are related with other unknown factors reminds to be elucidated in future experiments.

Furthermore, in the present experiment, animals provided with the herbal compound tended to present fewer aggressions and fights compared with pigs not supplemented, which might support the hypothesis that herbal compounds may have a sedative effect, reducing the number of aggressions, leading to less skin lesions as mentioned and probably diverting energy from negative social behaviour to growth. This hypothesis is reinforced by the higher body weight reported in chapter 3 and chapter 4 in the animals supplemented with the herbal compound.

The herbal compound did not influence meat or carcass quality, and consumers did not report any difference in acceptability.

3. PERCEPTION OF THE CONSUMERS

When the relative importance of three different attributes (feeding, production system, and price) was analysed by means of a conjoint analysis (chapter 4), the most determinant attribute for most of consumers was the production system, followed by feeding supplemented with herbs and the least considered attribute was the price. This finding is in line with the previously reported preference and increased willingness for animal friendly products reported in a study of Napolitano *et al.* (2010).

However, the importance of each attribute was not the same for all consumers, and three different clusters or groups of consumers were reported. One segment of consumers was mainly composed by men, for whom the most important factor was the price, with preference for lower prices. This results are in line with previous studies (e.g. Font-i-Furnols *et al.*, 2011). Furthermore, although the proportion of females of the other two clusters was not significantly different than the overall consumers' gender, both groups presented a higher proportion of females, which may explain the higher interest for animal welfare previously reported (Vanhonacker *et al.*, 2007).

Finally, higher prices and conventional production systems were considered as the most important factors for the smallest cluster of this experiment. Similar findings were reported by Sasaki *et al.* (2006). A possible hypothesis to explain this group of consumers may be the general perception that higher prices are related to a better product. However, a broader study with a higher number of consumers, a more balanced gender ratio, and more questions, would be required to better understand the different consumers of pork meat.

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Conclusions

CONCLUSIONS

1. Cortisol was detected in the hair of growing pigs but there were significant differences depending on the body region. These differences must be taken into account when sampling.

2. Salivary chomogranin A and hair cortisol had lower concentrations in those groups of growing pigs in which environmental enrichment and/or herbal compounds containing *Valeriana officinalis* and *Passiflora incarnata* were provided.

3. Assuming that environmental enrichment and herbal compounds reduced stress, the concentration of chomogranin A and hair cortisol were found to be useful tools to evaluate chronic stress in growing pigs.

4. Chromogranin A was more sensitive than hair cortisol to evaluate stress since higher and earlier differences were reported between the animals submitted to stress reduction and the control group

5. Environmental enrichment reduced abnormal behaviours such as stereotypies and redirected behaviour, while it increased activity and exploratory behaviour.

6. Herbal compounds containing *Valeriana officinalis* and *Passiflora incarnata* reduced negative interactions and skin lesions.

7. Environmental enrichment and herbal compounds increased body weight when treatments were applied during two months but meat and carcass quality were not affected.

8. An important segment of pork meat consumers showed a preference for systems aiming to increase the welfare. In general, women were more concerned about animal welfare, while for men, price was also a very important factor to consider.

9. According to the behavioural and physiological changes reported, environmental enrichment and herbal compounds enhanced the welfare of the animal and reduced stress in growing pigs.

“Alea jacta est”